The interaction between potato cyst nematodes and *Rhizoctonia solani* diseases in potatoes

Thesis

THE INTERACTION BETWEEN POTATO CYST NEMATODES AND *RHIZOCTONIA SOLANI* DISEASES IN POTATOES

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A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

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Declaration

This thesis was composed by the author and is a record of work carried out by him on an original line of research. All sources of information are shown in the texts and listed in the references; all help given by others is indicated in the acknowledgements.

None of this work has been presented in any previous application for any degree or qualification.

Signed.....(Matthew A. Back)

Abstract

The soil-borne pathogen *Rhizoctonia solani* and the potato cyst nematode *Globodera rostochiensis* are detrimental to the growth and productivity of potato crops in the UK. Previous work has shown that plant parasitic nematodes and fungal pathogens can occasionally interact synergistically to form destructive disease complexes. Furthermore, there is some evidence to suggest that an interaction might exist between *G. rostochiensis* and *R. solani* on potato.

In order to investigate interactions between *G. rostochiensis* and *R. solani*, a series of glasshouse and field experiments were undertaken (2000-2001). In field experiments, plots infested with similar population densities of *G. rostochiensis* were either uninoculated or inoculated with *R. solani*. A series of potato plant harvests were undertaken to investigate the effects of nematode infestation on the incidence and severity of *R. solani* diseases and the associated development of plants. In both experiments, a positive relationship was found between the density of *G. rostochiensis* juveniles present in potato roots and the incidence of stolons infected by *R. solani*, 6 weeks after planting. Weaker relationships were found between *G. rostochiensis* densities and stolon infections at 4 and 8 weeks after planting. In addition, a number of relationships were found between *G. rostochiensis* infestations and other *R. solani* disease symptoms, although these were less consistent across the harvest dates. In 2002, the experimental plot design was modified to further investigate the effect of *G. rostochiensis* on black scurf disease caused by *R. solani*. However, no relationship was observed between *G. rostochiensis* infestations and the later development of black scurf on daughter tubers.

The glasshouse experiments of this project did not show a direct interaction between *G. rostochiensis* and *R. solani* diseases. However, both glasshouse experiments were limited by the method used to infest potting medium with *G. rostochiensis*.

On the basis of results obtained from the field experiment, two controlled environment experiments (2002) were undertaken to investigate the growth rate of *R. solani* in response to root leachates from potato plants uninfested or infested with *G. rostochiensis*, at different time intervals after the introduction of nematodes. In addition, the concentration of carbohydrates and nitrogen was determined from the root leachate samples. In both experiments, *R. solani* isolates were found to have a significantly higher radial growth on media amended with root leachates from *G. rostochiensis* plants compared to uninfested plants at 12 days after the introduction of nematode treatments. Furthermore, a higher sucrose concentration was detected in root leachates from *G. rostochiensis* infested plants compared to root leachates from uninfested plants. Since exogenous carbohydrates are known to influence the growth and attraction of *R. solani*, these results may go some way to explain the interaction found.

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Statement of advanced studies

During the tenure of this project the author has published in a refereed journal and presented experimental findings at the scientific meetings detailed below.

Refereed papers

Back MA, Haydock PPJ, Jenkinson P, 2002. Disease complexes involving plant parasitic nematodes and soil-borne pathogens. *Plant Pathology* **51**, 683-97.

Conference presentations

a) Oral papers

Back MA, Jenkinson P, Haydock PPJ, 2001. A disease complex of potato involving the fungal pathogen *Rhizoctonia solani* (AG3) and the nematode *Globodera rostochiensis*. Presentation given at The Association of Applied Biologists – Offered Papers in Nematology, The Linnean Society, London, 11 December 2001.

Back MA, Jenkinson P, Haydock PPJ, 2002. Elucidation of the interaction between the fungal pathogen *Rhizoctonia solani* and the cyst nematode *Globodera rostochiensis* in potatoes. Presentation given at The British Society for Plant Pathology – Presidential meeting 2002 (Plant Pathology and Global Food Security), 8-10 July 2002.

Back MA, Jenkinson P, Haydock PPJ, 2003. Determination of the mechanisms involved in the *Globodera rostochiensis* – *Rhizoctonia solani* disease complex of potato. Presentation given at the 8th International Congress for Plant Pathology, Christchurch, New Zealand, 2-7 February 2003.

Conference proceedings (continued)

b) Poster papers

Back MA, Jenkinson P, Haydock PPJ, 2000. The interaction between potato cyst nematodes and *Rhizoctonia solani* diseases in potatoes. *Brighton Crop Protection Conference Pests and Diseases* 1, 503-6.

Back MA, Jenkinson P, Haydock PPJ, 2000. Field experimentation to investigate the interaction between *Globodera rostochiensis* and *Rhizoctonia solani* diseases in potatoes. Association of Applied Biologists – Offered Papers in Nematology, 12 December 2000.

Back MA, Jenkinson P, Haydock PPJ, 2002. The effect of granular nematicides on the development of *Rhizoctonia solani* diseases and their interaction with *Globodera rostochiensis* on potato. *Brighton Crop Protection Conference Pests and Diseases* 2, 777-80.

CONTENTS

CHAPTER 1.0 – INTRODUCTION

CHAPTER 2.0 – GENERAL METHODS

CHAPTER 3.0 – GLASSHOUSE EXPERIMENTS

CHAPTER 4.0 – FIELD EXPERIMENTS

CHAPTER 5.0 – CONTROLLED ENVIRONMENT EXPERIMENTS INVESTIGATING MODIFICATIONS IN THE POTATO RHIZOSPHERE DURING INFESTATIONS OF *GLOBODERA ROSTOCHIENSIS* AND SUBSEQUENT EFFECTS ON THE GROWTH AND DEVELOPMENT OF *RHIZOCTONIA SOLANI*

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER 1.0 – INTRODUCTION

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1.1 The pathogen *Rhizoctonia solani*

1.1.1 Introduction

Rhizoctonia solani Kühn (phylum: Basidiomycota, class: Hymenomycetes, order: Tulasnellales) is a soil-borne fungus found world-wide, which has been recorded to cause a variety of destructive diseases on a vast range of crop species (see section 1.1.5, Table 1.2). Ogoshi (1996) calculated that in Japan alone, 35 orders, 52 families, 125 genera and 142 species of plant were subject to infection by *R. solani*. The fungus can either exist as an anamorph (asexual, imperfect state) known as *Rhizoctonia solani* or as a teleomorph (sexual, perfect state) known as *Thanatephorus cucumeris*. *Rhizoctonia solani* is able to survive saprophytically (existing on dead organic matter), yet as previously mentioned, it is an effective pathogen of living tissue (biotroph).

1.1.2 Sources of inoculum

Figure 1.1 presents the lifecycle of the fungus *R. solani* on potatoes. From this diagram, it can be seen that *R. solani* can be spread by either seed or by soil-borne inoculum. Seed-borne inoculum consists of *R. solani* hyphae and sclerotia that are attached to the potato seed. There are numerous reports, which highlight the importance of infected seed in the development of *R. solani* diseases (Hide *et al.*, 1973; Gudmestad *et al.*, 1979; Adams *et al.*, 1980; Platt *et al.*, 1993). Carling *et al.* (1989) recorded that more than 90 % of plants produced from infected seed were subsequently affected by disease symptoms of *R. solani*.

Soil-borne inoculum consists of sclerotia and mycelium, which in the absence of a host, survive on plant debris in the soil (Sumner, 1996). The relative importance of soil-borne

inoculum is a much-debated subject in studies of *R. solani* epidemiology. Soil-borne inoculum, however, can be a significant factor in the infection of potato plants as observed by several workers (Frank & Leach, 1980; Scholte, 1987,89). Frank & Leach (1980) measured disease development on potatoes exposed to soil and seed-borne inoculum using glasshouse and field experiments. Both seed and soil-borne inoculum were found to infect plants, although seed-borne inoculum caused greater stem infections whilst soil-borne inoculum was responsible for greater stolon damage. These workers suggest that soil-borne inoculum of *R. solani* is more likely to attack stolons if it is located in the outer fringe of soil volume where stolons and roots occur. These results may reflect the attraction of *R. solani* to plant root exudates (section 1.1.3.1).

The length of time in which soil-borne *R. solani* is absent from a potato crop can affect the pathogenicity of the fungus. Scholte (1987) recorded that soil-borne *R. solani* caused fewer infections when potatoes were grown less frequently in crop rotations. A 1:5 crop rotation (maize, sugar beet, barley, barley, and potato) had a significantly lower incidence of stem infections than a 1:2 rotation (maize and potato or sugar beet and potato) or a continuous cropping of potato. Similarly, Carling & Leiner (1990a) found that the virulence of 71 isolates of *R. solani* (AG3) taken from a field of continuous cropping of potato and vegetable crops were higher than those taken from fields with crop rotation.

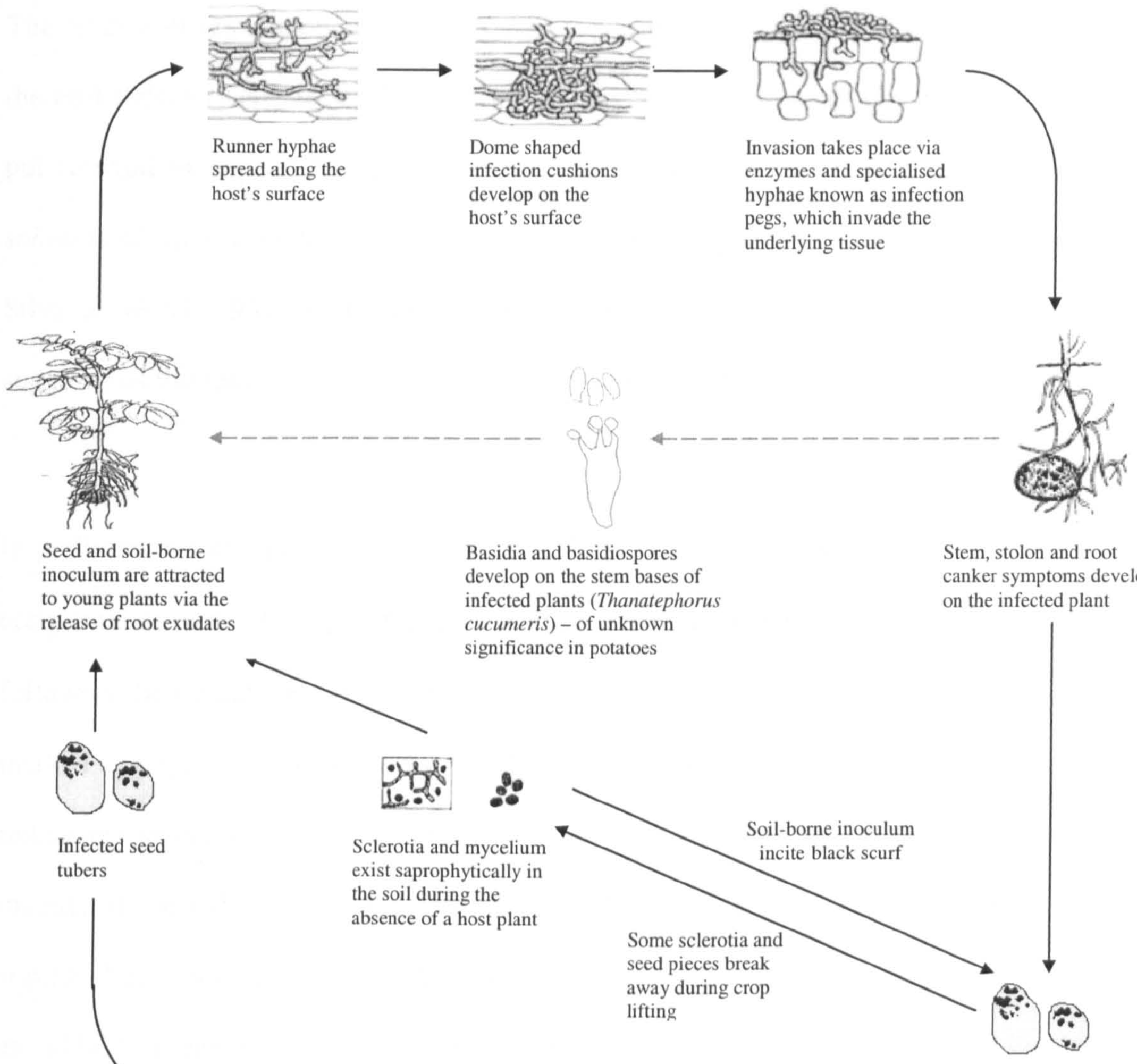


Figure 1.1 The life cycle of *Rhizoctonia solani* on potatoes (Parts of the diagram were reprinted from Plant Pathology, 3rd Edition, G.N. Agrios, page 490, Copyright 1988, with permission from Elsevier).

1.1.3 Rhizoctonia solani *infection of potato*

1.1.3.1 Attraction of *R. solani* to the host plant

The process of orientation used by *R. solani* to locate its host plant was first considered in the mid 1950's (Kerr, 1956; Flentje, 1957) and since then, several hypotheses have been put forward to explain this phenomenon. While some workers have suggested that *R. solani* is stimulated by the outer morphology of its host plant (thigmodifferentiation) (De Silva & Wood, 1964; Armentrout *et al.*, 1987), there is a greater body of evidence to suggest that the fungus is attracted by exudates emanating from the host.

In preliminary studies of *R. solani* attraction, Kerr (1956) observed how the fungus would conglomerate around 25 µm thick cellophane bags containing radish or lettuce seeds, following their incubation in soil inoculated with *R. solani*. Of particular interest, was the marked aggregation of *R. solani* hyphae that occurred directly opposite to the roots of both radish and lettuce seedlings. In comparison, empty cellophane bags incubated in *R. solani* inoculated soil did not become colonised by the fungus. Martinson (1965) also recorded a similar effect with seedlings of radish and bean in cellophane and nylon bags. In this study, an additional treatment of nylon bags containing concentrated seed exudates was also incubated in *R. solani* infested soil. Equally, this treatment was found to stimulate infection structures, but to a lesser extent than the bags containing germinating seeds. Although both of these studies (Kerr, 1956; Martinson, 1965) are rudimentary, they give an indication that *R. solani* is attracted by diffusible products from its host plant.

Reddy (1980) adopted an analytical approach to investigate the role of plant exudates in attracting *R. solani* to groundnut seedlings (*Arachis hypogaea*). Surface sterilised seedlings

of groundnut were inoculated with *R. solani* and examined 1, 2 and 3 weeks after germination. As the seedlings grew older, mycelial coverage on the hypocotyl surface became sparser and after 2 weeks, no fungal infection structures were detected. Samples of hypocotyl exudates were collected at weekly intervals so that they could be analysed for carbohydrates and proteins using chromatography and colorimetric methods. It was found that the quantity and array of amino acids, sugars and organic acids was significantly lower in those exudates collected from the older seedlings (2 and 3 weeks old). These measurements were related to fungal growth by taking dry weights of the mycelium at each sampling period. A decrease in the dry weight of *R. solani* mycelium was found to be relative to the reduction of the compounds present in the exudate.

1.1.3.2 Modes of penetration

Rhizoctonia solani invades its host in a number of ways, depending on the host species and the region of the plant where the infection takes place. There are several types of natural openings that occur in a plant's cuticle/epidermis such as stomata that can be readily exploited by the fungus (Dodman & Flentje, 1970; Chand *et al.*, 1985). However, the majority of studies report that *R. solani* penetration takes place with the aid of specialised infection structures. Flentje *et al.* (1963) observed how *R. solani* isolates attacking the root and stems of plants would form infection cushions (described below), whereas on aerial structures, *R. solani* invaded with lobate appresoria (short swollen branches originating from a single hypha).

Typically, *R. solani* invades the subterranean regions of potato plants using mycelial assemblages known as infection cushions (Chand *et al.*, 1985; Hofman & Jongebloed, 1988). The initial assemblage of an infection cushion begins with a shortening of hyphal

branches, the formation of many side branches close to one another, or continued growth that curls back and finally branches repeatedly (Dodman *et al.*, 1968b). This aggregation of side branches in the same area results in the formation of a discrete, tightly compacted, dome shaped infection cushion (Flentje 1957; Christou, 1962). According to Khadga *et al.* (1963) infection cushions can be formed through (i) an aggregation of short, stubby side branches from adjacent hyphae, (ii) an aggregation of side branches from a single hypha or (iii) terminal branching of a single hypha. Hoffman & Jongebloed (1988) examined potato sprouts infected with *R. solani* using scanning electron microscopy and found that infection cushions had formed from dense masses of short swollen cells belonging to highly branched secondary branches.

Penetration beneath the infection cushion is initiated by slender hyphae known as infection pegs (Christou, 1962; Fukutomi & Takada, 1979) invading the epidermal cells. Upon reaching the lumen of the epidermal cells, infection pegs give rise to a number of primary hyphae, which proliferate rapidly (Christou, 1962; Van Etten *et al.*, 1967). Hyphae may further colonise the surrounding cells and intercellular spaces of the cortical tissues, from which they frequently invade the vascular tissue and occasionally the pith (Van Etten *et al.*, 1967).

As previously mentioned, *R. solani* may utilise the natural openings of plants. Entry via stomata is generally considered a rare occurrence (Dodman *et al.*, 1968a; Marshall & Rush, 1980; Agrios, 1988) but takes place through a hypha entering a stomatal opening and filling a substomatal cavity before branch hyphae invade the tissue beneath (Dodman & Flentje, 1970; Chand *et al.* 1985). Ramsey (1917) reported that the lenticels of potato tubers could also become infected with *R. solani* whereby a 'dry plug' similar to that caused by wireworm damage developed. Dodman & Flentje (1970) state that *R. solani* may penetrate plants through wounds, which may be either associated with plant development

or injurious agents. For example Khadga *et al.* (1963) found that *R. solani* entered the host easily through injuries and quickly parasitised the underlying hypocotyl tissue of cotton. The association between nematode damage and *R. solani* infection will be discussed in section 1.3.2.1 of the literature review.

Evidently, some research has shown that invasion by *R. solani* may be assisted with the production of enzymes (Bateman, 1963a & 1963b; Van Etten *et al.*, 1967; Mall and Suresh, 1988) and/or phytotoxins (Frank & Francis, 1976). Amongst the various pectolytic and cellulolytic enzymes, extracted from culture filtrates of *R. solani*, polygalacturonse is recognised as being the most significant enzyme in relation to cellulose degradation and lesion formation in *R. solani* diseases (Bateman, 1963a, 1963b; Van Etten *et al.*, 1967; Mall & Suresh, 1988). Van Etten *et al.* (1967) generally found that polygalacturonase was concentrated in lesions caused by *R. solani* on bean (*Phaseolus vulgaris*) and had its peak activity during the earlier stages of lesion formation. Similarly, Mall and Suresh (1988) recorded a correlation between lower stem canker severity on potatoes and weaker endopolygalacturonase activity within the canker lesions. In addition to enzymes, phytotoxic substances such as phenylacetic acid (PAA) and its hydroxyl derivatives are reported to have a role in the pathogenesis of *R. solani* (Frank & Francis, 1976).

1.1.4 Symptoms and significance of R. solani *diseases of potatoes*

In the United Kingdom, *R. solani* is responsible for causing a number of diseases in the potato crop. The initial disease symptoms of *R. solani* occur soon after planting where the developing shoot tips become affected by sunken brown lesions. As the stem grows, these lesions often expand and coalesce with one another, spreading over the stem's entire circumference to form a necrotic collar or 'girdle' (Plate 1.1). The necrosis of the girdle may extend all the way through the tissue of the stem causing it to become severed or

'pruned' (Plate 1.2). The symptoms described above are widely known as stem canker (Read *et al.*, 1989; Hide & Horrocks, 1994; Simons & Gilligan, 1997b). In a similar manner stolons and roots can be infected and pruned by *R. solani* producing symptoms known as stolon (Plate 1.3) and root canker respectively. Tubers formed from stolons with necrotic lesions may develop a type of netted scab (Plate 1.4). This is caused by necrosis on the stolon tips being stretched over the surface of the tuber as it expands and grows (M.A. Back, personal observation). Stem canker is notoriously known for causing patchy and delayed emergence in crops (Read *et al.*, 1989), which is most likely to be the result of pruning damage on the developing stems. Research has consistently shown that stem and stolon canker can result in depressed tuber yields (Banville, 1989; Carling *et al.*, 1989; Hide *et al.*, 1989b). In addition these diseases cause a reduction in main stem number (Hide *et al.*, 1989a), a greater number of non-target seed sizes (Simons & Gilligan, 1997b), sessile tubers or 'little potato' (Plate 1.5)(Scholte, 1989), malformed tubers (Scholte, 1987) and tuber greening (Hide *et al.*, 1989a).

Basidiospores are produced during the sexual stage of the fungus (*T. cucumeris*) by fruiting bodies known as basidia. On potatoes this sporulation is seen as a whitish-grey collar at the stem base, just above the soil surface (Plate 1.6). Very little information, if any, is available on the significance of basidiospores of *T. cucumeris* in the potato crop. Basidiospores are important in the etiology of foliar *R. solani* diseases of crops such as rice (Naito, 1996).

Following plant senescence or the destruction of haulm, *R. solani* forms sclerotia on the daughter tubers, which appear as black 'tar' like encrustations and are widely known as 'black scurf' (Plate 1.7). The sclerotia are made up of layers of compact masses of barrel shaped moniloid cells (Sneh *et al.*, 1991). These tightly adhere to the tuber surface but do not generally penetrate much more than 5-6 cells into the periderm (Banville *et al.*, 1996). Nevertheless, Spencer & Fox (1979b) have recorded that black scurf can penetrate deeper,

sometimes reaching the cortex of tubers. Spencer & Fox (1979a) conducted a field experiment to examine the formation of black scurf in relation to haulm destruction and harvest date. When potato plots were desiccated in August, the incidence and severity of black scurf on daughter tubers was found to increase with time in contrast with plots left undesiccated. However, if plots were desiccated during September, the development of black scurf did not differ between desiccated and undessicated plots.

Dijst (1990) explored the mechanisms involved in black scurf formation. In this study the development of black scurf was recorded on cultures of *R. solani*, tubers and periderm strips, following exposure to sealed pots containing either healthy plants, plants with their haulm removed (topped), potting soil alone or perlite alone. In addition, these treatments were repeated with a layer of decaying stolons and roots. The results showed a significant increase in sclerotia development on each of the medium's tested, following exposure to plants, which had been topped. Plants still bearing foliage were found to slightly inhibit sclerotia development, whilst treatments of potting soil or perlite had no effect on sclerotia. The addition of rotting stolons and roots had no effect on sclerotium production. In a previous project (Dijst, 1988), this researcher came to the conclusion that water-soluble exudates of potato tubers were not responsible for black scurf initiation. Consequently, these results lead Dijst (1990) to suggest that sclerotia formation and inhibition were governed by volatile exudates released from the tubers. These gases, however, were unable to be fully determined in this work.

Although black scurf causes little physical damage to the tuber, the presence of black scurf on ware produce greatly reduces its saleability to pre-pack markets. In seed potatoes, the presence of black scurf can affect the classification awarded, since the sclerotia can initiate infections in successive crops. The 'Seed Potato Classification Scheme' for England and Wales states that no more than 3% of tubers tested should have a quarter of their surface area affected by black scurf in Super Elite, Elite, AA, CC stocks (Anon, 2003).

Plate 1.1 Girdling symptoms of stem canker on potato caused by *Rhizoctonia solani*

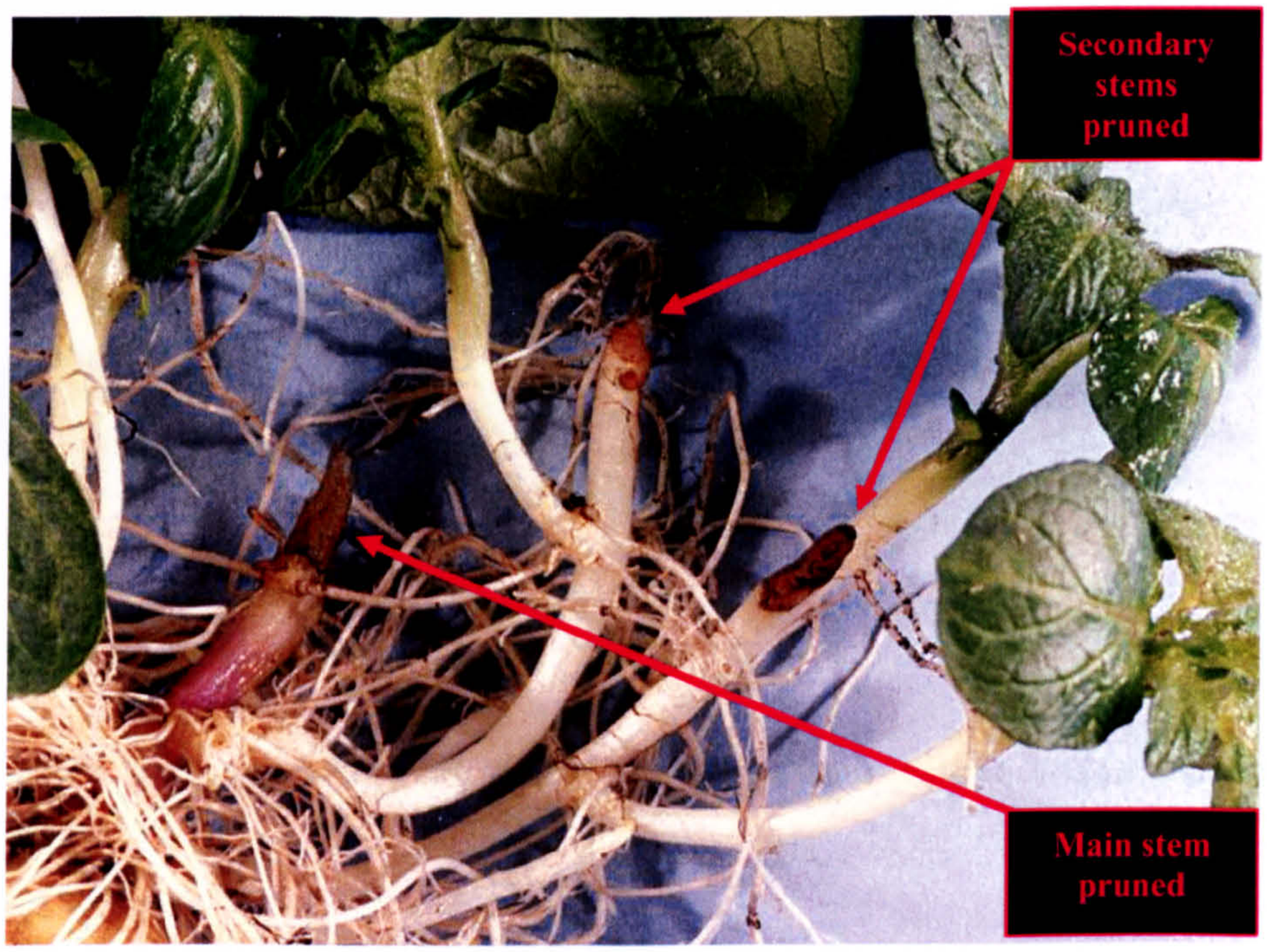


Plate 1.2 Main and secondary stem pruning on potato – a symptom of stem canker

Plate 1.3 Stolon canker symptoms on potato

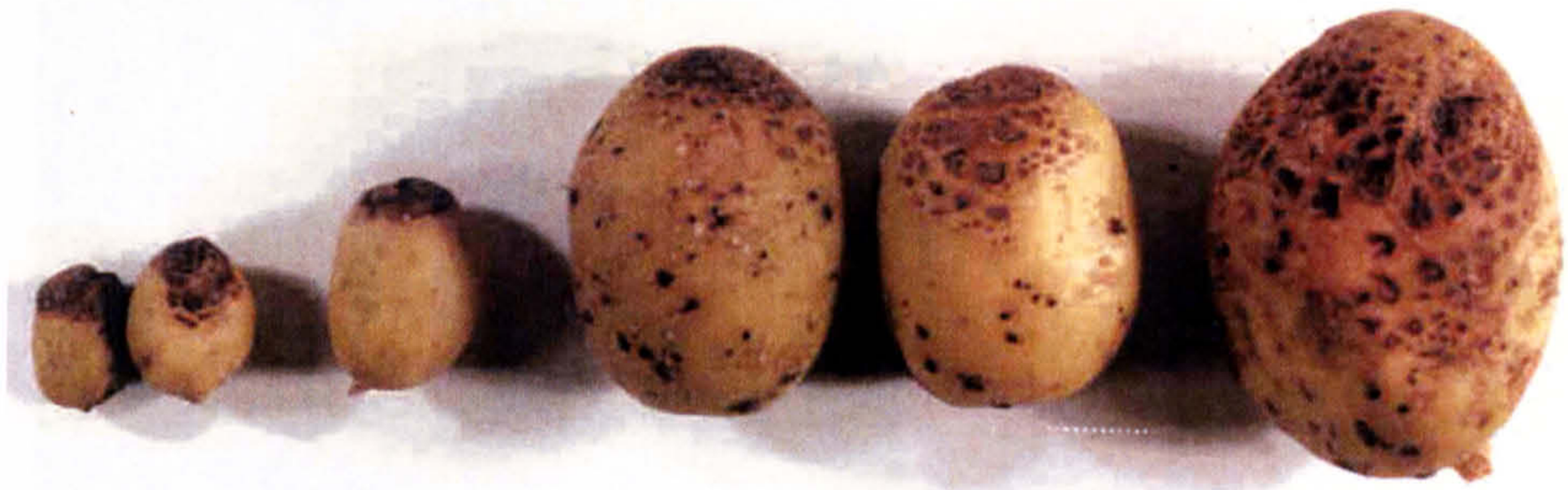


Plate 1.4 Netted scab on tubers of potato resulting from earlier stolon canker infections

Plate 1.5 Sessile tubers known as 'little potato' sometimes produced in *R. solani* infections

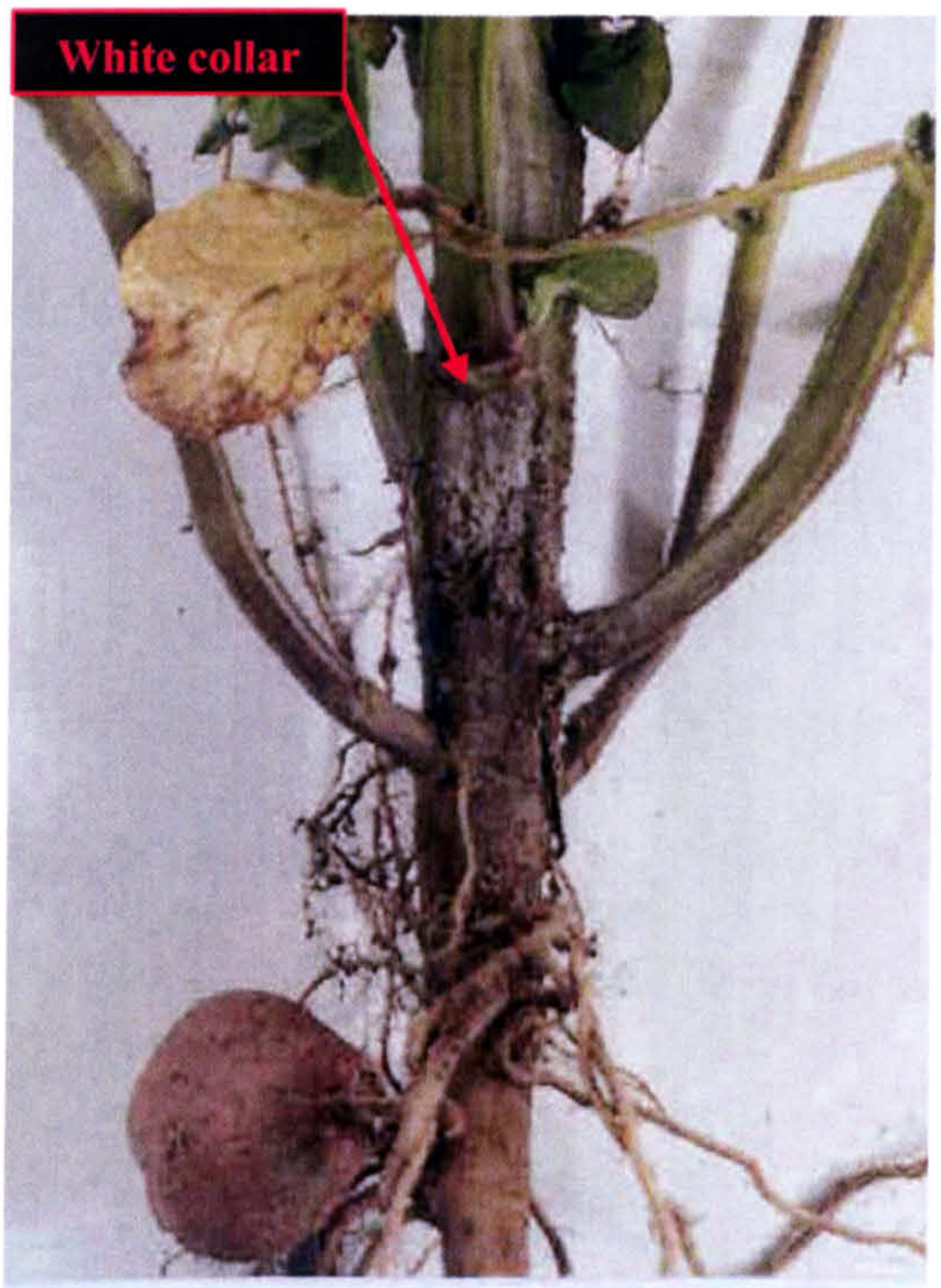


Plate 1.6 'White collar' produced by basidiospores of *Thanatephorus cucumeris* on potato

Plate 1.7 Sclerotia ('Black scurf') of *R. solani* on daughter tubers of potato

1.1.5 Anastomosis groups

Anastomosis = A natural connection between two tubular structures (Collins Concise Dictionary of the English Language, 1985)

Rhizoctonia solani is a large and highly heterogeneous species that is comprised of 13 subspecific groups (strains) (Carling *et al.*, 2001, 2002), which have been classified on their ability to undergo a process known as anastomosis. Anastomosis is the fusion of hyphae from two separate *R. solani* isolates. Consequently, anastomosis groups (AG's) are collections of closely related isolates. Carling *et al.* (1988) recognised that there were several degrees to the anastomosis reaction and subsequently produced a series of criteria (Table 1.1), which are frequently used by researchers to classify the reaction (Julián *et al.*, 1996; Cubeta & Vilgalys, 1997; Virgen-Calleros *et al.*, 2000).

In addition to the anastomosis reaction groupings, AG's 1, 2, 3, 4, 6, 7, 8 and 9 have been further divided into a number of sub-sets (Carling *et al.*, 2001) on the basis of contrasting morphology, pathogenicity, nutritional requirements, biochemical characteristics, molecular characteristics and DNA sequence (Ogoshi, 1987; Sneh *et al.*, 1991; Cubeta & Vigalys, 1997). For example AG 4 has been characterised into subgroups HG-1, HG-2 and HG-3 using DNA complementarity (Carling, 1996).

Table 1.1 Classification criteria for hyphal anastomosis between isolates of *R. solani* introduced by Carling *et al.* (1988)

Reaction category	Description of reaction	Genetic relatedness
C0: No interaction	Isolates of different anastomosis groups will grow over and underneath one another but will not make contact in any way (McCabe *et al.*, 1999).	Genetically distinct isolates belonging to the species *R. solani.*
C1: Contact	Contact occurs between hyphae without lysis of the cell wall - This is not considered to be anastomosis (Sneh *et al.*, 1991).	Distant relationship between isolates.
C2: Imperfect fusion (Killing reaction)	Fusion occurs between the cell walls and membranes of different isolates belonging to the same AG but with different vegetative compatibility's (Carling, 1996). Imperfect fusion results in cell death (Parmeter *et al.*, 1969) where plasmolysis occurs within 5-6 cells on either side of the fusion point (Flentje & Stretton, 1964; McCabe *et al.*, 1999).	Genetically distinct isolates belonging to the same AG.
C3: Perfect fusion	Complete fusion of cell walls and cytoplasm (cytoplasmic mixing). Isolates are of the same vegetatively compatible population (Carling, 1996). Continuous living cytoplasm exists through the fusion site (Sneh *et al.*, 1991)	Genetically identical or closely related isolates

Although anastomosis groups are not entirely host specific they can show fairly well defined tendencies (Agrios, 1988). Anastomosis group (AG) 3 is the most frequently recorded group to incite diseases on potato as indicated by findings from North America (Anderson, 1982; Bandy *et al*, 1988; Hill and Anderson, 1989), Northern Ireland (Chand and Logan, 1983), Japan (Ogoshi, 1987), Peru (Anguiz and Martin, 1989), Australia (Balali *et al*, 1995) and Mexico (Virgen-Calleros *et al*., 2000). In a recent UK census of *R. solani* isolates from potato, 101 out of 108 isolates were characterised as AG 3, whilst the remaining 7 were found to belong to AG 2-2 (J. Woodhall pers. com, unpublished data). Bandy *et al.* (1988) describes AG 3 as being the sole cause of stem canker and the major incitant of black scurf, while Chand & Logan (1983) observed that potatoes infected with AG 3 had higher stem canker and black scurf severities than AG 2-1. Nevertheless, AG's 1, 2-2, 2-1, 4, 5, 7, 8 and 9 have been reported to cause a range of disease symptoms on potato (Table 1.2). AG 8 only infects the roots of potato, causing root necrosis and pruning of main and lateral roots, which results in the stunted growth of plants (Hide & Firmager, 1990). Similar symptoms are also found on barley infected with *R. solani* AG 8 where the disorder is known as barley stunt disease. Table 1.2 provides an overview of the different AG groups recorded to produce disease symptoms on potato.

Table 1.2 Anastomosis groups of *R. solani* that cause diseases of potatoes and other crop species (Carling *et al.*, 1987, Carling & Leiner 1990b; Sneh *et al.*, 1991; Baird *et al.*, 1996; Banville *et al.*, 1996)

AG	Disease symptoms on potato	Reference	Additional hosts
1	Superficial lesions on stems, stolons and roots. Black scurf on tubers.	Carling & Leiner (1986, 1990b)	Rice (sheath blight) Soybean (web blight)
2-1	Small stem and stolon lesions. Black scurf on tubers.	Chand & Logan (1983) Carling & Leiner (1986)	Soybean (Seedling blight) Strawberry (bud rot)
2-2	Superficial lesion on stems, stolons and roots. Black scurf on tubers.	Carling & Leiner (1986, 1990b)	Crown and root rot (sugar beet) Bean (root rot)
3	Most isolates have moderate to high virulence on potato causing stem, stolon and root canker at temperatures ca. 10 °C. AG 3 is found to incite the majority of black scurf on tubers.	Chand & Logan (1983) Carling & Leiner (1986, 1990a & 1990b) Bandy *et al.* (1988)	Tobacco (leaf spot disease) Tomato (leaf blight)
4	Mild to moderate virulence on potato at temperatures ca.15-21°C. Stem and stolon canker.	Anguiz & Martin (1989) Carling & Leiner (1986, 1990a) Balali *et al.* (1995) Virgen-Calleros *et al.* (2000)	Sugar beet (crown rot) Tomato (fruit rot)
5	Mild to moderate virulence on potato at temperatures ca.15-21°C. Stem, stolon and root canker. Black scurf on tubers.	Bandy *et al.* (1988)	Turf grass (brown patch) Bean (root rot)
7	Superficial damage. Small lesions (<1 mm in diameter) form on shoots and roots at low temperatures (10 °C).	Carling & Brainard (1998)	Soybean (root rot) Cotton (root rot)
8	Root cankers causing pruning of lateral and secondary roots. Plants exhibit signs of stunted growth.	Hide & Firmager (1990) Carling & Leiner (1990b)	Wheat Barley (barley stunt disease)
9	Mildly parasitic on potato causing only minor lesions on the subterranean and aerial regions of stems.	Carling *et al.* (1987)	Lettuce, carrot (minor root lesions)

1.1.6 Optimal soil conditions for the development of R. solani *AG 3 diseases on potato*

According to published work, the severity of *R. solani* (AG 3) symptoms on potatoes is most severe at a temperature of 10°C (Carling & Leiner, 1990b; Kyritsis & Wale, 2002) and soil moistures of 40-45 % water holding capacity (Hide & Firmager, 1989; Kyritsis & Wale, 2002). Otten *et al.* (1999) suggested that *R. solani* invasion was additionally affected by the connectivity of air-filled pore spaces within soil. This study showed that at 'near' saturated soil conditions *R. solani* formed small dense colonies. In comparison, the fungus produced much larger colonies of lower biomass in soil conditions possessing highly connected air-filled pore volume.

1.2 Potato cyst nematodes

1.2.1 Introduction

The potato cyst nematodes (PCN) *Globodera rostochiensis* Wollenweber and *G. pallida* Stone (Family: Heteroderidae, Order: Tylenchida, Phylum: Nematoda) are amongst the most highly specialised of plant parasitic nematodes (Evans & Stone, 1977), having evolved close and intricate relationships with their principal host, the potato (*Solanum* spp.). Originating in the Andes Mountains of South America, PCN now have a world-wide distribution following their movement on potatoes to Europe (Evans & Trudgill, 1992). In the UK, a recent survey conducted by Minnis *et al.* (2000) showed that 64% of potato growing land was infested by PCN, where 67% contained pure *G. pallida* populations, 8% had pure *G. rostochiensis* and 25 % was infested by a mixture of the two species.

1.2.2 The life cycle of potato cyst nematodes

The life cycle of the potato cyst nematode (Figure 1.2) predominantly takes place in the roots of the potato plant although some studies have reported PCN using tubers (Hide & Read, 1991). Young juvenile nematodes (J2) (Plate 1.8) are attracted to the potato plant via gradients of root exudates, which diffuse from the plant into the soil (section 1.2.2.1). Upon reaching their hosts roots, the nematodes invade and migrate through the root tissue before initiating specialised feeding cells known as syncytia (section 1.2.2.3). Whilst feeding from the syncytia the nematodes remain sedentary and undergo several moults (J3, J4). The ratio of males to females formed is typically 1-1, though factors such as drought stress and overcrowding can lead to a higher proportion of males (Trudgill, 1967) because they require less time and nutrients to reach adulthood (Koenning & Sipes, 1998). Male nematodes develop within their cuticles during the third and fourth moult and emerge from

the roots as 'filiform' adults (J5) approximately 20 days after the juveniles first entered the roots (Mulder & Van der Wal, 1997). Meanwhile, the females enlarge during moults J3-4 until their bodies become so swollen that they rupture through the root's cortex as 0.5-0.75 mm spheres (Trudgill *et al.*, 1987). Male nematodes are attracted to the females through the release of sex pheromones (Green & Greet, 1972). The female's exposed vulva is fertilised by the male and subsequently the female's body fills up with eggs. Finally, the female dies but her outer cuticle tans and hardens to form a protective cyst (Plate 1.9) (Mulder & Van der Wal, 1997), which can contain anything in the region of 200-500 eggs (Brodie *et al.*, 1993). The eggs within the cyst will undergo a period of diapause, whereby hatching is delayed until there are favourable environmental conditions. In addition, a process known as quiescence can reverse the course of hatching to avoid unpredictable unsuitable conditions until favourable conditions return (Jones *et al.*, 1998). Hatching in *G. rostochiensis* and *G. pallida* is primarily stimulated by root exudates from their host plants but abiotic factors such as temperature (Franco, 1979) and pH (Jones *et al.*, 1998) can also play a role. In the absence of a host plant, cysts have been reported to remain viable in the soil for up to 20 years (Trudgill *et al.*, 1987).

1.2.3 Symptoms and significance of potato cyst nematodes

During PCN invasion and emergence, extensive damage is caused to the potato roots, reducing their ability to take up water and minerals. As a consequence plants are more prone to nutrient deficiencies, have a stunted growth (Plate 1.10), exhibit signs of drought stress, senesce prematurely and produce lower numbers of tubers. Evans & Stone (1977) estimated that PCN damage causes a 9 % loss of the annual potato yield in the UK, which has an approximate value of £43 M based on the mean value of the crop from 1990-95 (Haydock & Evans, 1998).

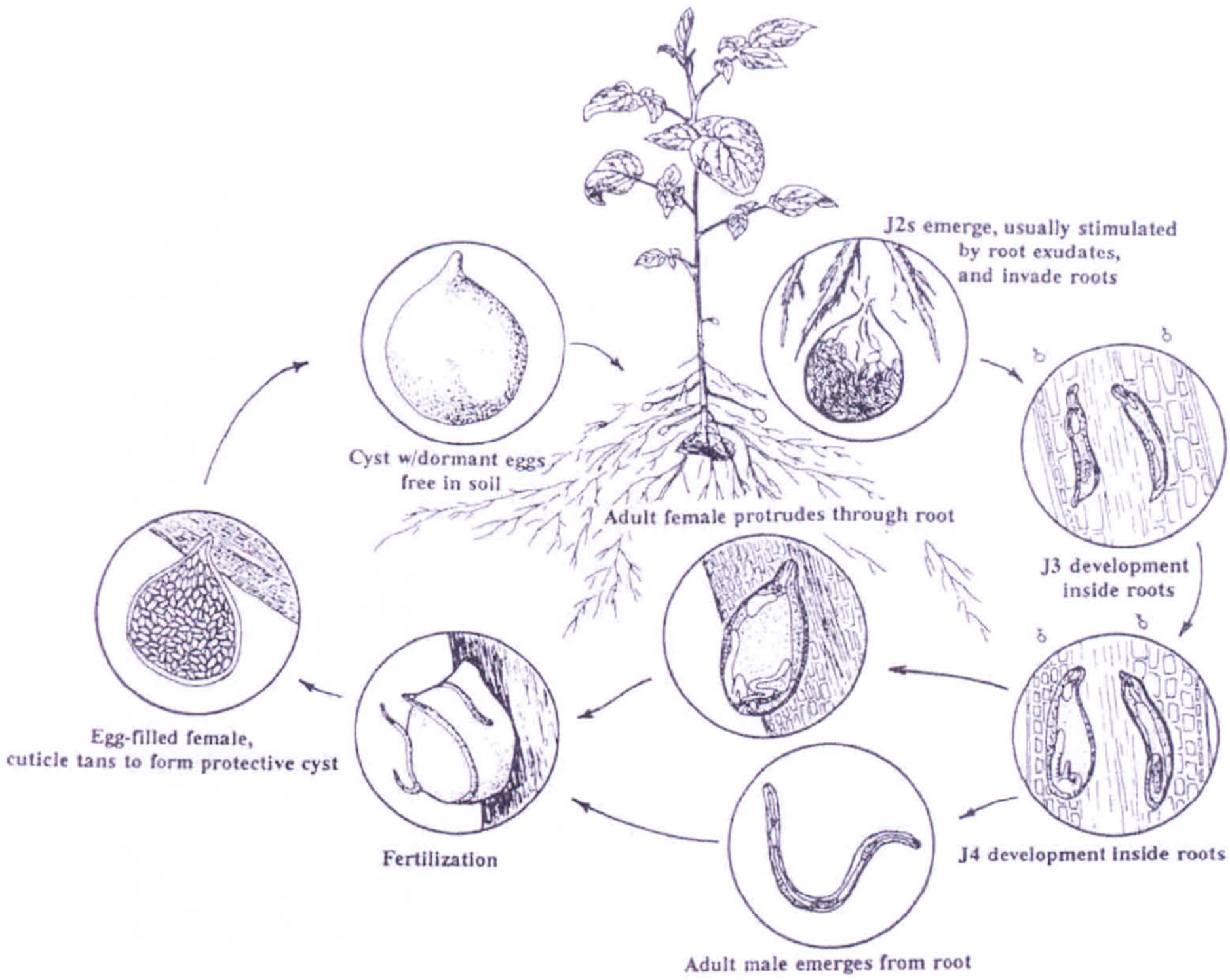


Figure 1.2 The life cycle of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* (reproduced with the kind permission of Professor Bill. B. Brodie)

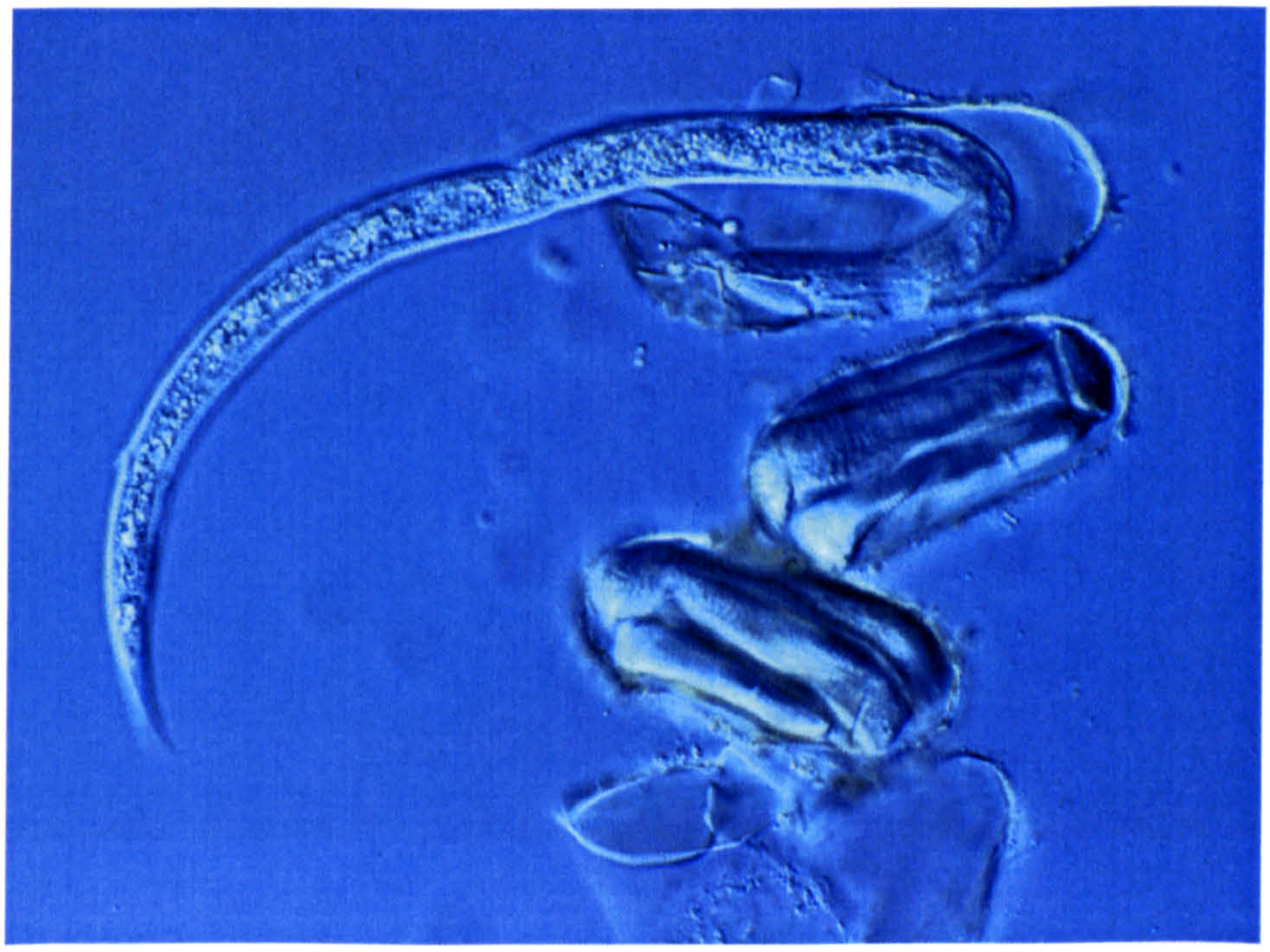


Plate 1.8 Juvenile *G. rostochiensis* (J2) emerging from egg case (plate reproduced U. Zunke - Nemapix, 1997)

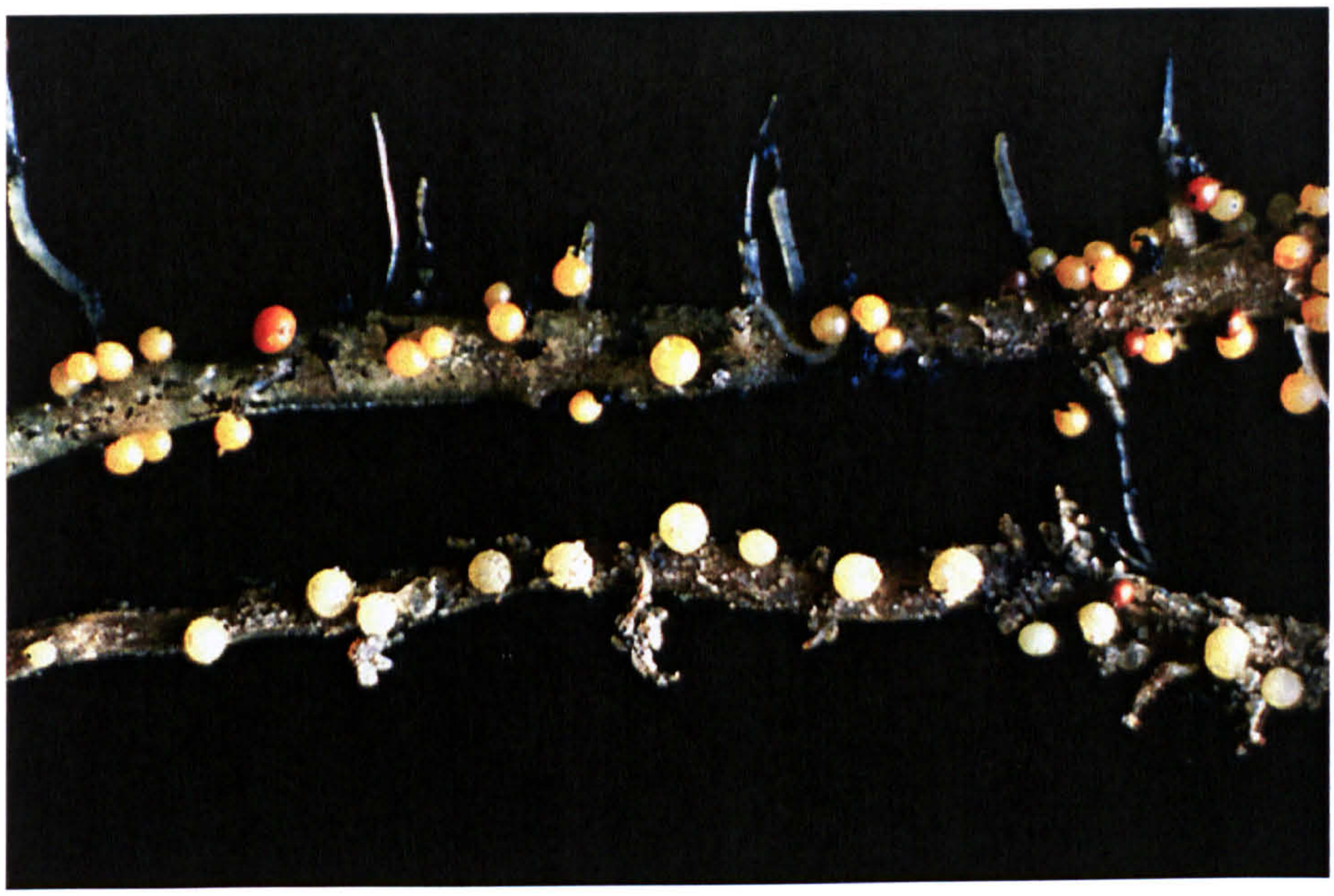


Plate 1.9 *Globodera* sp. cysts emerging from a potato root (plate reproduced U. Zunke - Nemapix, 1997)

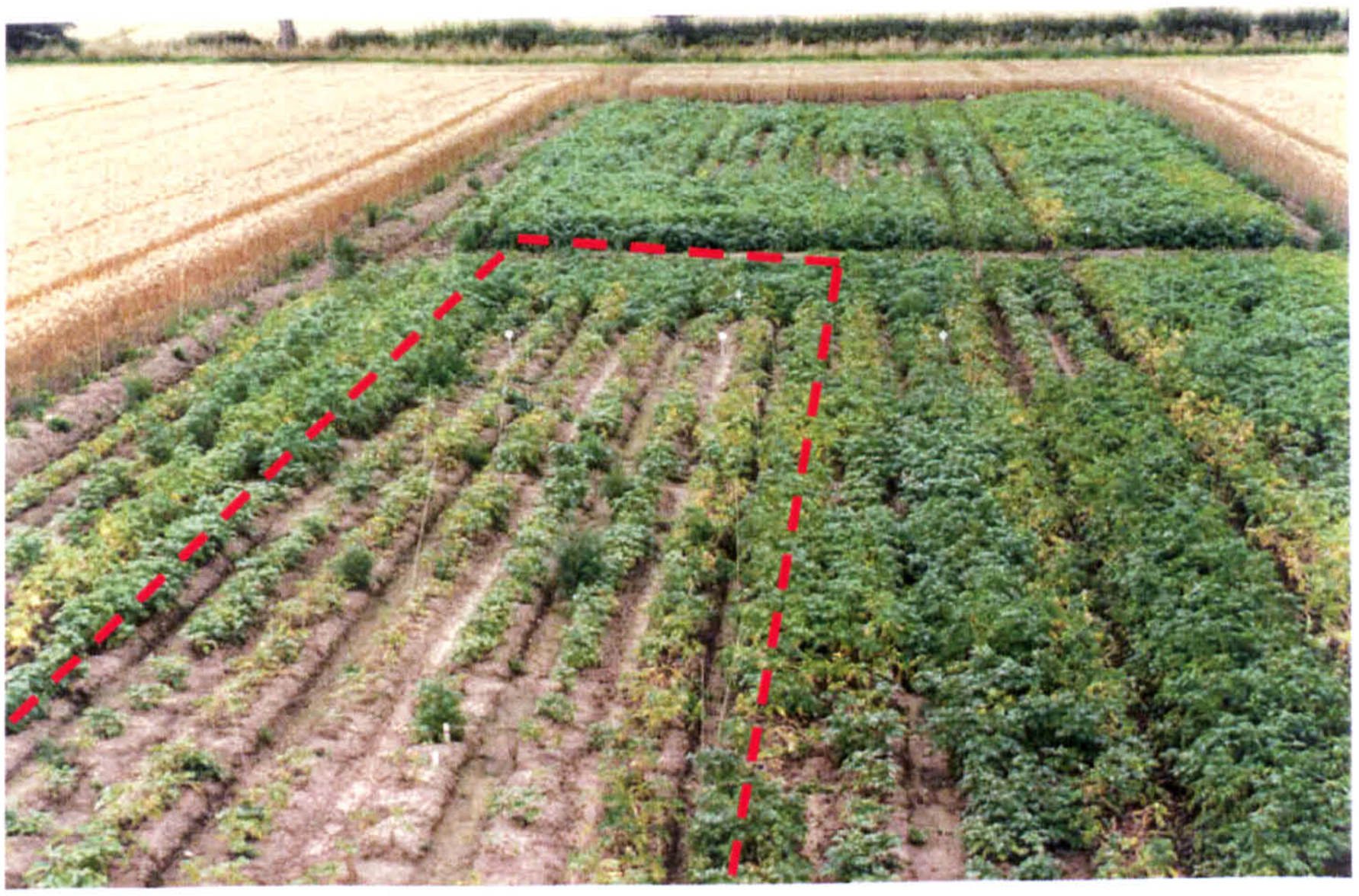


Plate 1.10 Potato plants infested with *Globodera rostochiensis* showing stunted growth and early senescence

1.2.4 Feeding biology

The feeding biology of potato cyst nematodes is particularly relevant to this project. In terms of interactions with soil-borne fungi such as *R. solani*, each component of the feeding process could play a significant role. For example, invasion tracts and feeding sites have been shown to be utilised by *R. solani* (Powell & Nusbaum, 1960; Polychronopoulos *et al.*, 1969). Conversely, fungi have also been recorded to influence the affinity of nematodes to plant roots.

1.2.4.1 Host finding

The attraction of plant parasitic nematodes such as *Globodera rostochiensis* to their plant hosts has received considerable attention, although the precise mechanisms involved are still under debate. Steiner (1925) first addressed nematode attraction, when he hypothesised that plant secretions facilitated the migration of plant parasitic nematodes to the roots of their host plants. Furthermore, he stated that the anatomical component employed for detecting the plant secretion stimulus were the amphids. The amphids are positioned at the anterior end of the nematode (head region) along with 16 other sensilla. Each are composed of a glandular sheath cell, a supporting socket cell and a number of dendritic processes bathed in secretions originating from the sheath cell (Perry, 1996).

Since Steiner's initial work, there is general agreement among authors that plant parasitic nematodes migrate to plants using a concentration gradient of some form (Bird, 1959; Klinger, 1965; Prot, 1980). With respect to the potato cyst nematode *G. rostochiensis*, investigations by Clarke & Hennessy (1984, 1987) provide a convincing explanation of how this attraction occurs. Potato root exudate (PRE) has long been known to stimulate hatching in *Globodera* spp. In earlier studies (Wallace, 1958; Weischer, 1959) it was

hypothesised that potato cyst nematodes orientated themselves to the roots of potato with diffusion gradients of PRE. Clarke & Hennessy (1984) tested the effect of PRE on the movement of *G. rostochiensis* by using sand columns (20mm high). Juvenile suspensions, containing approximately 200 juveniles were added to the top of the column, before being placed in sample jars containing 5ml of test solution. Following 24 hours incubation, the sand columns were removed and the number of juveniles that had passed into the sample jars counted. A significantly higher number of juveniles were found to pass through sand columns placed in jars containing 1:4 PRE compared to those placed in jars with glass distilled water (GDW) or artificial tap water (ATW). In addition to these findings, the authors found that juveniles of *G. rostochiensis* exposed to 1:4 PRE for 6 days had a significantly lower lipid and glycogen content to those maintained in GDW. These workers suggested that the reduction of such energy reserves, resulted from higher metabolic activity owing to increased motility in *G. rostochiensis*. Although this did not directly account for attraction to their plant host, it indicated that *G. rostochiensis* second-stage juvenile's (J2) were able to detect and were stimulated by PRE.

Clarke & Hennessy (1987) found many of the components of PRE responsible for stimulating hatching in *G. rostochiensis* were also effective attractants. These hatching factors were identified as the ions; Ba^{2+}, La^{3+}, Zn^{2+} and the organic acids; picrolonic acid, cinchomeronic acid, nicotinic acid and picric acid.

More recently, Rolfe *et al.* (2000) used electrophysiological apparatus to examine the sensitivity of *G. rostochiensis* juveniles to PRE and semiochemicals. A glass suction pipette was used to hold the anterior region of a freshly harvested J2, whilst test solutions were applied with a micro-syringe via a 'perfusion capillary' within the pipette. Measurements of electrical activity were recorded with an electrode inserted into the anterior of the nematode at the end of the suction pipette. It was found that the electrical

activity or spike activity (mean spikes per second) of J2's increased significantly during exposure to PRE. As the PRE was replaced with artificial tap water, spike activity began to decrease. When exudates from sugar-beet plants were tested in the same manner, there was no response in electrical activity suggesting that *G. rostochiensis* was able to distinguish between exudates from host and non-host plants. This work further illustrates the sensitivity of J2 *G. rostochiensis* to PRE and supports the original notion of Steiner (1925).

A good proportion of research on nematode attraction has focussed on gradients of PRE, although it is important to note the wide scope of additional reports detailing attraction to other factors. For example, attraction of plant parasitic nematodes has been recorded with CO_2 gradients (Bird, 1960; Klinger, 1965), thermal gradients (Rode, 1970; Dusenbury, 1996), redox potentials (Bird, 1959) and pH (Prot, 1980). It is quite feasible that a number of these factors could play a role in nematode orientation.

1.2.4.2 Selection of an invasion site, penetration and migration

On arrival at the roots, potato cyst nematodes migrate to the region just behind the developing root tips (Evans & Stone 1977) and sometimes in the area of lateral root formation (Golinowski *et al.*, 1997). Widdowson *et al*, (1958) suggested that this might be the result of higher PRE release in this area, though there is currently no experimental evidence to support this hypothesis. The process by which tylenchid nematodes select a site for penetration is described in the account of Doncaster & Seymour (1973), who used filmed microscopy to make their observations. In summary, juveniles of *Globodera* spp. were found to stop moving except at the anterior end when contact was made with the root. The root surface was explored with intermittent stylet (nematode feeding apparatus) probing whilst the lip region was pressed and/or rubbed against the surface. Once a penetration site was selected by a nematode, it began rhythmic, harder and quicker stylet

thrusts at the same spot on the root surface until the surface was penetrated. Following penetration, the stylet was removed and another site was selected close to the first and the stylet thrusting began again. This sequence was repeated until a group of nematode-created perforations amalgamated to form a single slit. Subsequently, the nematode could enter the epidermal cell by applying pressure with its head. Once inside the root, nematodes migrate through the cortex, cutting through further cell walls until they reach the vascular cylinder where specialised feeding sites are assembled (Hussey & Grunder, 1998).

1.2.4.3 Syncytia induction and feeding

Cyst nematodes (*Globodera*/*Heterodera* spp.) extract nutrients from plants by inducing specialised feeding cells, known as syncytia within the pericycle, endodermis or adjacent cortex of the root (Jones, 1981). There is very little literature available on the induction of syncytia in *Globodera* spp. Nevertheless, Wyss & Zunke (1986) have intensively assessed the behaviour of the closely related sugar-beet cyst nematode *Heterodera schachtii* inside the roots of *Brassica napus* seedlings. Using high-resolution video-enhanced contrast recording techniques, these workers observed the initial stages of syncytial development and nematode feeding. This process began with J2's selecting a cell within or close to the vascular cylinder and then using thrusting stylet movements to penetrate it. Following penetration, the stylet was inserted 3-4 μm into the cell lumen where it then ceased to move. Following this, the nematode was observed to start feeding by pumping regularly with its metacorpus (muscular pumping organ). During nematode feeding a number of changes takes place in the initial syncytial cell. The cell itself expands and partial cell wall dissolution occurs, allowing neighbouring protoplasts to fuse with one another to produce a single multinucleate cell (Wyss, 1988). In addition, the cytoplasmic density increases with

the production of ribosomes, whilst mitochondria and Golgi bodies proliferate (Jones, 1981).

1.3 Interactions between plant parasitic nematodes and soil-borne pathogens

1.3.1 Introduction

The natural soil environment harbours a multitude of microorganisms. As many as 10^6 – 10^8 bacterial cells, 10^6–10^7 actinomycete cells, 5 x 10^4– 10^6 fungal colony forming units (CFU), 10^5– 10^6 protozoa and 10^4–5 x 10^5 algae were estimated to be present in a gram of field soil taken from the surface (Gottlieb, 1976) while Richards (1976) found c. 1 x 10^7 nematodes from an area of $1m^2$ of fertile soil. Although many of these organisms are saprophytic, having little, if any effect on cultivated crops, the moist soil environment is favourable for the activities of plant parasitic nematodes and for the growth and multiplication of pathogenic fungi. It is of no surprise, therefore, that a whole variety of inter-relationships between these organisms have been demonstrated.

It has long been understood that the development of disease symptoms is not solely determined by the pathogen responsible, but is dependent on the complex inter-relationship between host, pathogen and prevailing environmental conditions. In addition, in nature plants are rarely, if ever, subject to the influence of only one potential pathogen. This is especially true of soil-borne pathogens where there is tremendous scope for them to interact with many other microorganisms occupying the same ecological niche. Disease aetiology and the reasons for the multi-factorial nature of disease causation are described by Wallace (1978).

Examples of interaction between soil microbes influencing disease development can be seen in plant parasitic nematode – pathogen complexes. A disease complex is produced through a synergistic interaction between two organisms. Synergistic interactions can be summarised as being positive where an association between nematode and pathogen results

in plant damage exceeding the sum of individual damage by pest and pathogen (1 + 1 > 2). Conversely, where an association between nematode and fungus results in plant damage, which is less than that expected by the sum of the individual organisms, the interaction may be described as antagonistic (1 + 1 < 2). Where nematodes and fungi are know to interact and are shown to cause plant damage which equates to the sum of individual damage by pest and pathogen, the association may be described as neutral (1 + 1 = 2). Although the former two associations can be readily demonstrated experimentally, the latter can prove difficult to identify since neutral associations can result in similar plant damage to that seen in additive associations where nematode and pathogen are known not to interact with one another.

The first recorded case of a nematode-fungal interaction was made by Atkinson (1892), who observed that fusarium wilt of cotton (caused by *Fusarium oxysporum* f. sp. *vasinfectum*) was more severe in the presence of root-knot nematodes (*Meloidogyne* spp.). Since these initial observations, interactions involving nematodes and fungi have been documented on a vast range of crop species (Pitcher, 1965; Powell, 1971; Mai & Abawi, 1987; Evans & Haydock, 1993; Back *et al.*, 2002). In particular, the fungus *R. solani* has been frequently found to interact with endoparasitic nematodes as shown in Table 1.3. In the subsequent sections of this review, the mechanisms behind interactions involving plant parasitic nematodes and soil-borne fungi will be discussed with focus on those involving *R. solani*.

Table 1.3 Examples of interactions involving endoparasitic nematodes and *R. solani*

Nematode	Crop	Reference
Globodera rostochiensis	Potato	Back *et al.* (2000)
Heterodera avenae	Wheat	Meagher & Chambers (1971)
Heterodera glycines	Soyabean	Dave (1975)
Heterodera schachtii	Sugar beet	Polychronopoulos *et al.* (1969)
Meloidogyne hapla	Radish	Wajid Khan & Muller (1982)
Meloidogyne incognita	Tomato	Arya & Saxena (1999)
Meloidogyne incognita	Tobacco	Batten & Powell (1971)
Meloidogyne javanica	Peanut	Abdel-Momen & Starr (1998)
Pratylenchus thornei	Chickpea	Bhatt & Vadhera (1997)

1.3.2 Mechanisms underlying synergistic interactions

1.3.2.1 Utilisation of nematode induced wounds by soil-borne fungi

Depending on specific life cycles, plant parasitic nematodes are able to cause a variety of types of wound on host plant roots whilst entering or feeding. For example, ectoparasitic nematodes such as *Trichodorus* spp. and *Tylenchorhynchus* spp. feed upon root epidermal cells, leaving simple micro-puncture type wounds behind. In contrast, the specialised feeding strategies of sedentary endoparasites (*Meloidogyne* spp., *Globodera* spp. and *Heterodera* spp.) can cause far greater damage to plant roots. Such damage occurs during nematode invasion, occupation and emergence of the roots (section 1.2.4).

Several authors (Bergeson, 1972; Taylor, 1990) have regarded nematode invasion sites and tracts as being inconsequential in the aetiology of fungal diseases, however, there are a number of reports that clearly illustrate that nematode damage has a role in the establishment and development of disease caused by fungal pathogens. Histological studies appear to be the key to unravelling the association between fungal pathogens and the injuries caused to plants by plant parasitic nematodes. This is particularly highlighted in the work of Polychronopoulos *et al.* (1969), where the invasion process of *Heterodera schachtii* (beet-cyst nematode) was found to facilitate the infection of sugar beet (*Beta vulgaris*) by the fungus *R. solani*. During their investigation, sugar beet seedlings grown in either nematode-infested or nematode-free soil were exposed to *R. solani* before being examined microscopically over a series of 12-hour intervals for three days. On inspection of the seedlings 36 hours after inoculation, distinct differences could be seen between the two treatments. When in combination with *H. schachtii*, the hyphae of *R. solani* were found to grow vigorously through the epidermis and cortex. Closer examination showed that hyphal colonisation frequently followed tracts made by invading nematode juveniles.

On the epidermal surfaces of the seedlings, the pathogen was found to produce fewer infection cushions in the presence of nematodes than when it was present alone. These authors suggested that infection cushion synthesis could have been hindered in some way by the invading nematodes. However, nematode invasion sites may provide *R. solani* with the necessary portals for penetration and entry, consequently reducing the need for developing more sophisticated infection structures such as infection cushions. In a similar way, *R. solani* is known to exploit natural openings on the outer surfaces of plants such as stomata (Chand *et al.*, 1985) and lenticels on potato tubers (Ramsey, 1917) to invade underlying tissue.

In addition to the cavities caused during invasion, nematodes produce other forms of mechanical damage to plant roots that are open to exploitation by soil-borne fungi. Fagbenle & Inskeep (1987) used scanning electron microscopy to study concomitant infections of *Meloidogyne hapla* and *Rhizoctonia solani* on peanut (*Arachis hypogaea*). Eleven weeks after inoculation with *R. solani*, the root galls were often found to be split, leaving a rough surface comprised of cortical cells, which rapidly became colonised by *R. solani*. However it is unclear whether the cracks of the galled roots aided the penetration of *R. solani*.

In order for female cyst nematodes to reproduce, the females/cysts must rupture through the root cortex to allow the vermiform males to fertilise them. This event often produces a number of cracks and crevices where the swollen female has emerged. Several authors have suggested that these openings might be used by opportunistic pathogens to reach the underlying tissue of roots more easily (Bergeson, 1972; Evans & Haydock, 1993; Golden & Van Gundy, 1975).

It seems surprising that there are relatively few reports to demonstrate fungal utilisation of nematode induced wounds, particularly where sedentary endoparasites are involved. In order to validate this hypothesis it would appear that positive quantitative data coupled with convincing histological evidence is required. Future work in this area might benefit from using equipment such as time-lapse and video-enhanced light microscopy together with some form of image analysis system. Video-enhanced light microscopy has previously been used for studying nematodes (Wyss & Zunke, 1986) and fungi (McCabe *et al.*, 1999). The application of image analysis systems could help overcome the problems of quantifying the density of fungal pathogens in regions of nematode damage.

1.3.2.2 Nematode induced physiological changes to the host plant

The feeding sites of sedentary endoparasitic nematodes (giant cells or syncytia) are zones of high metabolic activity, having a greater number of Golgi apparatus and mitochondria, whilst the cytoplasm is more dense and contains many ribosomes (Jones, 1981). According to Taylor (1979,1990) and Abawi & Chen (1998), syncytia and giant cells contain higher amounts of total protein, amino acids, lipids, DNA and sugars. It is, therefore, no surprise that these nutrient rich cells should be frequently recorded to be the chosen substrate for fungal colonisation (Meléndez & Powell, 1970; McLean & Lawrence, 1993; Wajid Khan & Muller, 1982; Abdel-Momen & Starr, 1998). Perhaps the most comprehensive studies into such localised nematode induced modifications are those of Golden & Van Gundy (1975). In their field studies with okra and tomato, fumigants were applied to field plots to reduce *M. incognita* and *R. solani* (ethylene dibromide and methyl bromide, respectively) and create independent and combined treatments. In untreated plots (i.e. plots with both *M. incognita* and *R. solani*), *R. solani* was isolated from the galls of *M. incognita* a week after gall formation. Two weeks later, numerous black sclerotia were found encrusted to the galls. In contrast, sclerotia were absent from un-galled regions of the roots. Four weeks

following gall formation, substantial root decay occurred. Furthermore, histological sections revealed that *R. solani* had penetrated root cells from the sclerotia attached from the gall surfaces. *Rhizoctonia solani* appeared to have a marked trophic intercellular pattern through the cortex of galled roots towards nematode induced giant cells. Wajid Khan & Muller (1982) reported similar observations with *M. hapla* and *R. solani* on radish. Galls infected by *R. solani* had abundant sclerotia on their surfaces while giant cells were colonised extensively by hyphae. Similarly, Abdel-Momen & Starr (1998) found that a reduction in the pod yield of peanut was significantly greater in co-infections of *M. javanica* and *R. solani* where a concentration of fungal growth was found around the galled regions.

Cyst nematodes also form nutrient rich syncytia for the purpose of development. Histological studies of *H. schachtii* infested sugar beet seedlings exposed to *R. solani* indicated that syncytia were a more favourable substrate to the fungus than normal cells (Polychronopoulos *et al.*, 1969). The authors also describe how the syncytium appeared to be a suitable 'food base' for the colonisation of other tissues by the fungus. Hyphae were seen to spread from the syncytia to the corticovascular tissue, which had not been invaded by nematodes.

As well as these localised effects, some authors (Bowman & Bloom, 1966; Batten & Powell, 1971; Hillocks, 1986) have supported the notion that nematode induced physiological changes can be systemic. In such cases the nematode induced beneficial factors or substances to fungi are hypothesised to be translocatable within the plant (Wajid Khan, 1993). Bowman & Bloom (1966) and Hillocks (1986) whilst investigating this process employed a 'split-root' technique, whereby the root system of the plant of interest was bisected into two separate containers. The first root half was infested with the interacting nematode species, whilst the second half was inoculated with the interacting

fungal pathogen. Bowman & Bloom (1966) infested one half of a tomato plants root system with nematodes (*M. incognita*) and the other half with *Fusarium oxysporum* f. sp. *lycopersici*. Their results revealed that disease development on plants was dependent on being exposed to both *M. incognita* and the fungus. Regardless of the results, these split-root experiments do not identify systemic physiological modifications or in fact any other mode of interaction. They simply indicate that an interaction might exist. Indeed, it could be equally feasible to conclude that the effects seen were a result of nematode occupation causing a loss of resistance or plant stress. Further studies involving critical biochemical analysis of plant material taken from plants either infested or uninfested with nematodes would significantly increase our understanding of nematode induced systemic change. For example, the nutritional quality of plants infested with plant parasitic nematodes may prove more favourable to fungal pathogens. By determining the nutritional requirements of the fungus and quantifying these metabolites in nematode infested and uninfested plants, a clearer picture could be attained. Equally, plant parasitic nematode infestations may well reduce the quantities of fungitoxic compounds.

1.3.2.3 Modifications within the rhizosphere

The release of plant root exudates is considered to be an important factor in the attraction of both soil-borne fungi (Flentje, 1957; Reddy, 1980; Grayston *et al.*, 1997) and plant parasitic nematodes (Klinger, 1965; Clarke & Hennessy, 1987). There are a number of ways in which plant parasitic nematodes might influence the release of root exudates and thus alter the subsequent response of soil-borne pathogens. Firstly, the damage inflicted on plant roots during the process of plant parasitic nematode invasion could well result in greater volumes of root exudates attractive to fungal invaders. Secondly, certain potato cultivars have been shown to produce an increase in the number of lateral roots in response to invasion by potato cyst nematodes (Evans & Stone, 1977). Such an increase in root

surface area may well give rise to a higher production of root exudates. Finally, plant parasitic nematode infestation may influence the chemical profile of the root exudates released making them more favourable to fungal pathogens (Bergeson, 1972).

Perhaps the most classic examples of this process are those involving root-knot nematodes. The aggregation of fungi, primarily *R. solani*, around root-knot galls of many plants has drawn attention to changes occurring within the rhizosphere. Golden & Van Gundy (1972) made preliminary observations of this effect during their studies of the *M. incognita-R. solani* complex of tomato. Tomato roots infested with *M. incognita* were seen to become more susceptible to fungal attack by *R. solani* with increasing age. By adopting the cellophane membrane techniques of Kerr (1956) and Flentje (1957) they observed that *R. solani* would aggregate on cellophane, which was directly opposed to the galled regions of the roots. In contrast, un-galled areas only received sparse mycelial coverage. In a later publication, Golden & Van Gundy (1975) undertook further studies with semi-permeable membranes (cellophane) on tomato and okra, infested with *M. incognita*. Introduction of *R. solani* (via mycelial plugs) to the external surfaces of the cellophane once again produced sclerotia opposite the galls of *M. incognita*. Microscopic examination of the sclerotia showed that their formation consisted of irregular branching and inter-winding to form loosely constructed, undifferentiated structures. From these studies the authors concluded that metabolic leakage from the galls of *M. incognita* might well explain the elevated attraction of *R. solani*.

Van Gundy *et al.* (1977) undertook an extensive investigation using tomato to evaluate the hypothesis regarding metabolic leakage. Firstly a technique known as 'double-root' was utilised, whereby a secondary root system was induced to allow experimentation on the natural or primary root system. The attraction of *R. solani* to nematode infested plants was facilitated by the use of a hydroponic system to remove root leachates. Tomato plants

either infested with *M. incognita*, exposed to *R. solani*, infested with *M. incognita* and exposed to *R. solani* or left untreated, were found to be free of root-necrosis after 5 weeks under the hydroponic regime. However, when leachates taken from *M. incognita* infested roots were applied to plants exposed to *R. solani* alone, necrosis developed. Conversely, treatment of *R. solani* treated plants with leachate from untreated plants did not result in root-necrosis. Furthermore, it was found that if the experiment was repeated in the absence of the hydroponic system, plants would only develop root-rot when exposed to both organisms. The results of these studies implied that *M. incognita* infested plants were producing some form of attractant for *R. solani*. Consequently, the researchers proceeded to examine the properties of exudates emanating from the nematode infested roots. Nematode-infected plants were found to have higher levels of carbon-14 (^{14}C) metabolites. During the time of sclerotial development 14-21 days following nematode invasion, the major constituents of the ^{14}C labelled metabolites were determined as nitrogenous compounds, such as amino acids and proteins: such nitrogenous compounds are important in the virulence of *R. solani* (Weinhold *et al.*, 1972).

1.3.2.4 Reduction of host resistance

In the development of crop species that express resistance to economically important pests and diseases, the significance of nematode-fungus complexes is seldom reported and yet there are a number of studies that report breakdown of resistance during concomitant infections (Sidhu & Webster, 1977; Marley & Hillocks, 1994; France & Abawi, 1994; Uma Maheswari *et al.*, 1995,1997; Vargas *et al.*, 1996). Previously, Bergeson (1972) commented, "it is frustrating to the plant breeder to see the fruits of his labour come to naught by the nematode-fungus conspiracy".

Typically, loss of resistance has been tested with the application of split-root methods as described earlier. Investigators have adopted this type of approach to determine whether the loss of pathogen resistance induced by nematode infestation occurs as a result of the breakdown of a systemic chemical defence system within the host plant. Bowman & Bloom (1966) found that the tomato cvs. Rutgers and Homestead, previously resistant to *F. oxysporum* f. sp. *lycopersici* developed symptoms of wilt during split-root experiments with *M. incognita*. Further studies (Sidhu & Webster, 1977) using root layering and grafting techniques confirmed that a nematode induced factor could be passed through a resistant scion (a graft from a resistant tomato cultivar) and render it susceptible to *F. oxysporum* f. sp. *lycopersici*. In contrast resistant scions in tomato plants free from *M. incognita* infestation could block infection by *F. oxysporum* f. sp. *lycopersici*.

Very few examples of resistance loss are available for disease complexes involving root-rot fungi such as *R. solani*. However, Khan & Husain (1989) have reported resistance loss to *R. solani* in several cowpea cultivars, during infestation by *M. incognita*.

Interactions between species of nematodes and fungi can vary considerably over plant species cultivars and lines as indicated in studies of disease complexes on multiple crop genotypes (Khan & Husain, 1989; Uma Maheswari *et al.*, 1995; Abd-El-Alim *et al.*, 1999). Consequently, some investigations have been unable to demonstrate resistance loss (Jones *et al.*, 1976; Castillo *et al.*, 1998; Johnson & Santo, 2001) whilst other reports have shown the converse with identical combinations of nematode and fungus species. There are also other abiotic factors such as soil type and temperature, which have been shown to affect interactions (Uma Maheswari *et al.*, 1997) and these may have varied between individual studies on specific disease complexes.

1.3.2.5 Pathogen induced changes to the host plant

Just as nematode activity can increase the severity of diseases caused by fungal pathogens so can nematode populations be elevated during concomitant infections with root infecting pathogens (Vrain, 1987; Taheri *et al.*, 1994). Whilst there are far fewer reports of such a phenomenon several hypotheses have been proposed. Faulkner & Skotland (1965) observed that *Pratylenchus minyus* reached its reproductive peak at the same time as the maximum expression of wilt disease (*Verticillium dahliae* f. sp. *menthae*) on peppermint plants (*Mentha piperita*). The authors suggested that *V. dahliae* may produce root growth promoting substances, such as indole-3-acetic acid (IAA), as previously recorded for *V. albo-atrum* (Pegg & Selman, 1959) resulting in an enlarged root system, releasing greater volumes of root exudate, and thereby attracting more plant parasitic nematodes (Clarke & Hennessy, 1987; Rolfe *et al.*, 2000). Some studies illustrate how plant roots, infected with fungal pathogens can be more attractive to plant parasitic nematodes. For example, Nordmeyer & Sikora (1983) considered how the attraction of *Heterodera daverti* might be affected by *Fusarium avenaceum* infected clover (*Trifolium subterraneum*) seedlings when compared to uninfected plants. *In-vitro* experiments showed that a significantly greater proportion of *H. daverti* migrated towards diffusates from *F. avenaceum* infected clover roots than towards diffusates from healthy plants.

The influence of fungi on plant parasitic nematode orientation has also been investigated by Edmunds & Mai (1967), who showed that *Pratylenchus penetrans* conglomerated around a CO_2 source under *in-vitro* conditions in agreement with the previous findings of Bird (1959) and Klinger (1965). CO_2 measurements taken from alfalfa plants (*Medicago sativa*) infected with *Trichoderma viride*, and in particularly *Fusarium oxysporum*, were considerably higher than those found in healthy roots. Elevated levels of CO_2 from infected plants may have contributed to the increased attraction of *P. penetrans* towards alfalfa

roots, previously seen in earlier experiments (Edmunds & Mai (1966a, 1966b). Whether or not CO_2 emissions would produce a similar nematode response within the natural soil environment remains open to debate and subject to further experimentation. Moreover, the subject of nematode orientation is still largely unknown in the field of plant nematology.

Some research has suggested that fungal infections cause a deterioration or breakdown of plant resistance to nematode attack. Hasan (1985) encountered this effect during routine field screening of chilli pepper cultivars and lines, which under controlled glasshouse conditions, had shown promising resistance against *M. incognita.* In this case, resistance was lost in 2 out of 5 previously fully resistant and 8 out of 16 previously moderately resistant lines. Furthermore, individual plants subject to resistance loss expressed symptoms of collar rot and damping off diseases. The disease inducing fungal pathogens were isolated and positively identified as being *R. solani* and *Pythium aphanidermatum.* Subsequently, a glasshouse experiment was devised to test the effect of *R. solani* and *P. aphanidermatum* on the resistance of cv. Jawala (resistant) and cv. Longthin Faizibadi (moderately resistant) to *M. incognita.* On both cultivars, the presence of *R. solani* or *P. aphanidermatum* caused a significant increase in the reproductive capacity (number of egg masses and eggs produced) of *M. incognita.* In terms of resistance, the rating of Jawala and Longthin Faizibadi was demoted to moderately resistant and susceptible, respectively. The exact nature of this mechanism was not determined but the activities of pathogen produced enzymes may have compromised the physical barrier conferred on the resistant chilli lines or chemical defences such as the anti-feedant proteinase inhibitors, described by Lilley *et al.* (1999) may well have been disrupted during nematode infections. New technology such as proteomics, may provide a better understanding of such interactions but in the meantime the interactions between plant parasitic nematodes and soil-borne pathogens need to be considered in future plant-breeding programmes

1.3.3 Other considerations

1.3.3.1 Biotic factors

So far the nematode-pathogen complex has only been discussed with reference to the type and level of damage caused to the plant host. However, this is not the only outcome to arise from these synergistic relationships, since either nematode or fungus can be indirectly affected during their cohabitation of a mutual host. Direct antagonistic interactions, which involve the parasitism of nematodes by soil-borne fungi, have been extensively studied and reviewed (Kerry, 2000). The indirect effects that fungi exert upon nematodes in disease complexes are less well known, yet remain important in terms of future nematode multiplication.

Earlier, work was described where feeding sites of sedentary endoparasites were shown to be preferable substrates for plant pathogenic fungi (Polychronopoulos *et al.*, 1969; Negrón & Acosta, 1989; Abdel-Momen & Starr, 1998). In all of these investigations, the nematode syncytia or giant cells were disrupted or damaged to some extent during fungal colonisation. Fattah & Webster (1983) have monitored changes within *M. incognita* giant cells during co-infection of tomato roots with *Fusarium oxysporum* f. sp. *lycopersici* and *Meloidogyne incognita.* Transmission electron microscopy of root tissue, three weeks after infection with *Fusarium*, revealed that the giant cells of *M. incognita* were smaller and spherical. Chromatin within the nuclei of these cells had condensed along the nuclear membrane, which was partially fragmented and swollen. In contrast the giant cells of plants inoculated with *M. incognita* alone were much larger, had well defined membranes, large nuclei and dense cytoplasm. These workers stated that successful assemblage and maintenance of giant cells is vital for the growth and reproduction of root-knot nematodes, and that destruction of large proportions of giant cells will result in the premature death of

female nematodes. Consequently, these types of disturbances are likely to affect the development of subsequent nematode populations.

In addition to fungal disruption of nematode feeding sites, plants affected by disease complexes may be more prone to early senescence and death (Griffin *et al.*, 1993; Walker *et al.*, 1998), which in turn may prevent nematodes from completing their life-cycles. Competition for nutrients (Jorgenson, 1970) or root space (Ketudat, 1969) may be responsible for the decline of nematode populations, though these concepts appear to be difficult to demonstrate.

1.3.3.2 Abiotic factors

As previously mentioned, fluctuating environmental parameters are often found to affect one or more of the interacting organisms in a disease complex. Temperature has been recorded to be critical in some nematode-fungus interactions (France & Abawi, 1994; Walker *et al.*, 2000) but not in others (Griffin *et al.*, 1993; Uma Maheswari *et al.*, 1997; Walker *et al.*, 1999). Interestingly, France & Abawi (1994) observed that a genotype of bean with dual resistance against *Fusarium oxysporum* f. sp. *phaseoli* and *Meloidogyne incognita* would exhibit visible wilt symptoms if both organisms were inoculated together at 27°C. It was suggested that the high temperature caused a breakdown of resistance to *M. incognita* and in turn the nematodes broke resistance to the wilt-inducing pathogen.

Soil type has been shown to have no influence over disease complexes involving *Meloidogyne hapla* and *Phytophthora megasperma* f. sp. *medicaginis* on alfalfa and *R. solani* and *M. javanica* on soybean (Griffin *et al.*, 1993; Agu & Ogbuji, 2000). Conversely, Uma Maheswari *et al.* (1997) stated that soil type can affect interactions between *F. oxysporum* f. sp. *ciceri* and *M. javanica* on chickpeas. According to their findings, the

development of wilt disease by *F. oxysporum* f. sp. *ciceri* was higher in a clay soil (48% clay) whilst *M. javanica* caused greater plant damage in a loamy sand. On the basis of these results, heavier textured soils appear to be unsuitable for root-knot nematodes and, therefore, the establishment of disease complexes with *F. oxysporum* f. sp. *ciceri* would be improbable. From the many studies on nematode and fungal epidemiology, it seems highly plausible that additional environmental factors such as soil pH (Rupe *et al.*, 1999), soil moisture and meteorological conditions (Robinson *et al.*, 1987) will also be linked to the development of a disease complex depending on the organisms involved. The impact of abiotic factors on nematodes and soil-borne pathogens emphasises the importance of undertaking field experiments to test for synergistic interactions. Evans & Haydock (1993) discussed the validity of pot-based and laboratory experiments. They argue that the 'acid test' for determining how agriculturally significant an interaction is, depends upon field experimentation.

1.3.4. Previous research investigating interactions between Rhizoctonia solani *and potato cyst nematodes*

During the mid-1920's, a possible association between *R. solani* infection and potato cyst nematodes (then known as potato eelworm) was noted on potato crops in South Lincolnshire (Morgan, 1925; 1926). Morgan (1925) reported that PCN cysts appeared to be in equal abundance with diseases such as *R. solani* and later described (Morgan, 1926) how potato roots were often found smothered with cysts and sclerotia of *R. solani*. In a later study by Grainger & Clark (1963), this relationship was investigated under glasshouse conditions. Here, surface sterilised potato tubers (cv. Epicure) were planted in sterilised soil, which had subsequently been infested with *Heterodera rostochiensis* (syn. *Globodera* spp.) using c. 0.9 cysts g^{-1} soil, inoculated with *R. solani*, infested with *H. rostochiensis* and inoculated with *R. solani* or left untreated. Tuber yield measurements taken at the end of the experiment revealed no significant differences between pots treated with *H. rostochiensis* or *R. solani* alone and those left untreated. However, in pots where both organisms were present, the yield was depressed by approximately 33%. When fresh tubers were replanted in the pots and soils from the first experiment, a similar but accentuated response was obtained suggesting that both fungal and nematode densities had increased.

Dunn & Hughes (1964, 1967) and more recently Mazurkiewicz-Zapalowicz & Waker-Wójciuk (1994) have also observed a reduction in plant growth (tomato and potato respectively) during concomitant infections of *R. solani* and PCN. Conversely, the results of Stelter & Meinl (1967) indicated that *R. solani* and PCN act independently of one another as opposed to being involved in a disease complex. These workers found that the yield of potato plants (cv. Gerlinde) grown in pots was unaffected by *R. solani* alone, but was reduced by 60 % during PCN infestations. Stelter & Meinl (1967) argued that

additional infection by *R. solani* in pots infested by PCN occurred as a result of additive effects and not synergism between the two organisms.

Yield and plant development are not the only parameters affected during the co-habitance of *R. solani* and *G. rostochiensis* on potato. Mazurkiewicz-Zapalowicz & Waker-Wójciuk (1994) have recorded intense 'necrosis-like changes' on the potato cultivar Mila hosting *R. solani* and *G. rostochiensis*. In comparison, disease symptoms on plants exposed to *R. solani* alone were relatively superficial. Furthermore, plants co-inoculated with the pathogen *Botrytis cinerea* (grey mould) and *G. rostochiensis* did not develop accentuated disease symptoms, suggesting that a specific mechanism could have been involved in interactions between *R. solani* and *G. rostochiensis*.

Synergistic interactions involving *R. solani* have also been recorded with several other species of cyst nematode. For example a reduction in plant height and root length together with severe root browning and stunting was found during combined infestations of cereal cyst nematode (*Heterodera avenae*) and *R. solani* on wheat plants (cv. Insignia) (Meagher & Chambers, 1971; Meagher *et al.*, 1978). Furthermore, Polychronopoulos *et al.* (1969) provided good histological evidence for a synergistic interaction between the beet cyst nematode (*H. schachtii*) and *R. solani* (section 1.3.2.1).

As well as synergistic relationships, several authors have described antagonism between *R. solani* and *G. rostochiensis*. For instance, Stelter and Meini (1967) recorded a reduction (c.25 %) in the final populations (Pf) of *G. rostochiensis* during exposure to *R. solani* under glasshouse conditions. Similarly, Janowicz *et al.* (1994) has also observed marked reductions (c.34 %) in the Pf densities of *G. rostochiensis* (cysts, eggs and larvae) in soil colonised by *R. solani*. Here an estimation of the viable and non-viable eggs and larvae was determined by staining the cyst contents with 0.05% aqueous cotton blue in

lactophenol. Healthy eggs/larvae appeared unstained while dead eggs/larvae took up the stain allowing a viability percentage to be calculated. Although microscopic examination of eggs, cysts and larvae showed no apparent evidence of fungal invasion, the dead larvae appeared contorted and lacking in turgor suggesting that an enzyme or phytotoxin used by *R. solani* could have affected them. The toxic effect of fungal filtrates upon plant parasitic nematodes has also been observed elsewhere (James, 1966; Chen *et al.*, 2000).

1.4 Aims of the project

The aim of this project was to determine whether relationships between *Globodera rostochiensis* and *Rhizoctonia solani* exist and if appropriate, to assess their significance in potato production. Previous research, investigating interactions between these organisms has presented a mixed picture, where synergism, antagonism and no interaction have been described. This may in part be due to the fact that many of these studies have been conducted on a small scale using a low number of replicate treatments (Grainger & Clark, 1963; Mazurkiewicz-Zapalowicz & Waker-Wójciuk, 1994). However, the fundamental weakness of these investigations is that none have sought to examine relationships between *R. solani* and *G. rostochiensis* under field conditions. Consequently, this project has focussed on glasshouse and field experiments to fully investigate the interaction. The objective of the glasshouse experiments was to assess potato plant development and *R. solani* disease severity following independent and combined inoculation treatments of *G. rostochiensis* and *R. solani*. In field experiments, plots infested with similar population densities of *G. rostochiensis* were either uninoculated or inoculated with *R. solani* to investigate the effects of nematode infestation on the incidence and severity of *R. solani* diseases and the associated development of plants.

Having found an interaction between pest and pathogen, a series of controlled environment and laboratory studies were undertaken to examine root exudation in *G. rostochiensis* infested potatoes in relation to the growth of *R. solani*. *In vitro* experiments were conducted to compare the growth of *R. solani* isolates on either media amended with leachates from potato plants infested with *G. rostochiensis* or media amended with leachates from potato plants uninfested with *G. rostochiensis*. Leachates collected from potato plants either infested or uninfested with *G. rostochiensis* were analysed for carbohydrate and nitrogen content.

CHAPTER 2.0 – GENERAL METHODS

CHAPTER 2.0 GENERAL METHODS

2.1 General methods for working with *Rhizoctonia solani*

2.1.1 Basic culturing and identification of R. solani

All culturing work was undertaken under aseptic conditions in a laminar flow cabinet to minimise contamination from microorganisms. Before undertaking any operations, the working surfaces were thoroughly swabbed with 80% industrial methylated spirit (IMS) and a Bunsen burner was lit to reduce the probability of contamination from air-borne microorganisms. Instruments, such as cork borers and dissecting needles were sterilised in the flame of the Bunsen burner before and after each operation as were the necks and caps of glassware containing media. Spillages were immediately swabbed with 80% IMS and the contaminated materials were placed in biohazard bags for autoclaving. A laboratory coat was worn during the culturing of *R. solani* and the laminar flow cabinet was sterilised with 80% IMS upon completion of the work.

Five isolates of *R. solani* were initiated with sclerotia (black scurf) of *R. solani* originating from potato tubers (cv. Maris Piper). The sclerotia were surface sterilised by submerging each piece of sclerotium in a Petri dish containing 1 % sodium hyperchlorite for ten minutes. Following this, the sclerotia were momentarily dried on tissue paper before being transferred to the centre of a Petri dish (plate) containing 1% water agar (WA) amended with streptomycin sulphate (Appendix 1). Agar plates were produced by pouring the molten agar mix (autoclaved for 20 minutes at 121°C and cooled at room temperature for ca. 40 minutes to 45-55°C) into sterile disposable Petri dishes. Once the plates had been

inoculated with the sclerotia they were sealed with parafilm ®, inverted and placed in an incubator at 15±2°C for 7 days.

After incubation, sub-cultures were made from the plates inoculated with sclerotia. Plates were selected on the basis of having no visible contaminants such as *Penicillium* spp. *Rhizopus* spp. or *Mucor* spp. The plates were examined under a binocular microscope (Magnification = x 30) to check for the diagnostic features of *R. solani*. According to the current species concept (Parmeter & Whitney, 1970; Sneh *et al.*, 1991) *R. solani* can be characterised by: -

a) Some shade of brown hyphal pigmentation

b) Branching near the distal septum of cells in young vegetative hyphae

c) Constriction of hyphae and formation of septa a short distance from the point of origin of hyphal branches (Plate 2.1)

d) Dolipore septa

e) Multi-nucleate cells in young vegetative hyphae - staining hyphae with safranin O can be used to visualise nuclei (Bandoni, 1979)

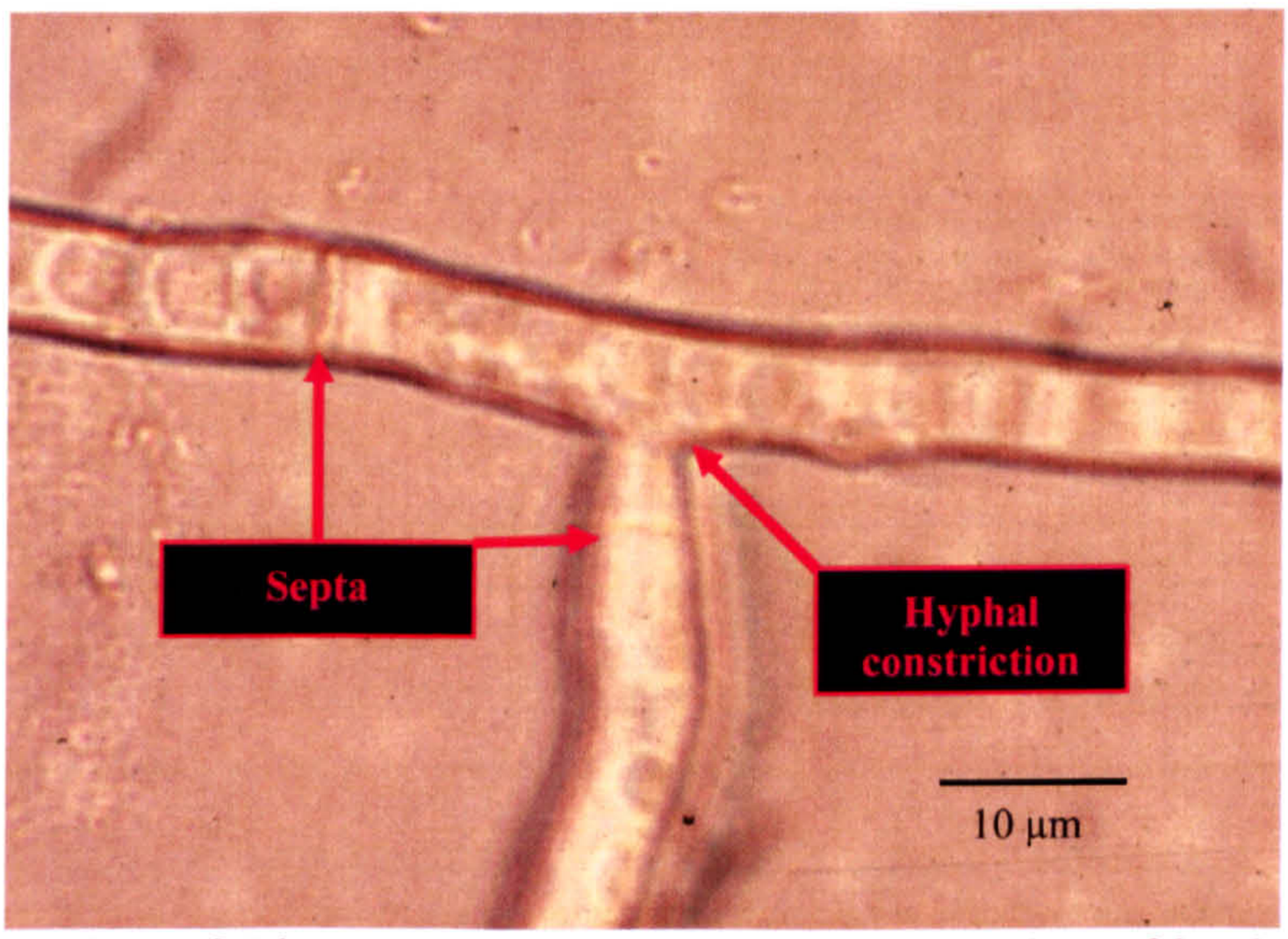


Plate 2.1 Hyphae of *Rhizoctonia solani* showing constriction of hyphae and septa a short distance from the point of origin of hyphal branches

Once *R. solani* was identified on the sclerotia-inoculated plates, 6 mm plugs were cut from the growing margin of the colony with a No. 3 cork borer and placed in the centre of Petri dishes containing potato dextrose agar (PDA) (Appendix 1). PDA plates inoculated with the mycelial plugs of *R. solani* were incubated at 15±2°C for 7 days after which they could be used for bulking up inoculum for experiments (section 2.1.4) or inoculating media for long term storage (section 2.1.3). Cultures of *R. solani* on PDA could be kept up to 6 weeks after inoculation before being sub-cultured onto fresh media. Excessive sub-culturing was avoided by returning to long term cultures or by passing the fungus through potato plants and re-isolating from infected stem lesions.

2.1.2 Determination of R. solani anastomosis groups

As previously outlined in section 1.1.5 (page 15) the fungus *R. solani* is a species complex comprised of 13 or more anastomosis groups. There are several methodologies which can be used for determining the anastomosis group of an unknown isolate (Tu *et al.*, 1969; Parmeter *et al.*, 1969; Castro *et al.*, 1988) but the clean slide method of Kronland & Staghellini (1988) was found to be the easiest application. 'Tester' isolates of anastomosis groups known to infect potatoes (AG 2-1, AG 3, AG 4, AG 5, AG 8) were obtained from the Scottish Crop Research Institute (SCRI). Most studies indicate that *R. solani* AG 3 is the most frequent cause of *R. solani* diseases in potato (Bandy *et al.*, 1988) and therefore the AG 3 isolate was initially tested with unknown isolates. Mycelial plugs (6 mm) were taken from both the unknown and the tester isolates and placed approximately 25 mm apart on a sterile microscope slide. Slides were placed in Petri dishes lined with moist filter paper (Whatman No. 1 ®) and incubated for 24-48 hours at 15±2°C or until the hyphae of each plug just made contact with one another (Figure 2.1). The plugs were then removed by cutting them away with a scalpel and a drop of both safranin O and 3% KOH was pipetted onto the interface between the two isolates before a cover slip was placed over them. The prepared slides were examined under a light microscope (magnification = x 200) where C2-C3 hyphal fusion points (Carling, 1988) were sought between isolates (Plate 2.2). If 3 points of fusion were found on 3 separate slides, the anastomosis group was successfully determined. Care was taken to avoid false positives or points of 'self-anastomosis' (Plate 2.3) by following hyphae to their point of origin.

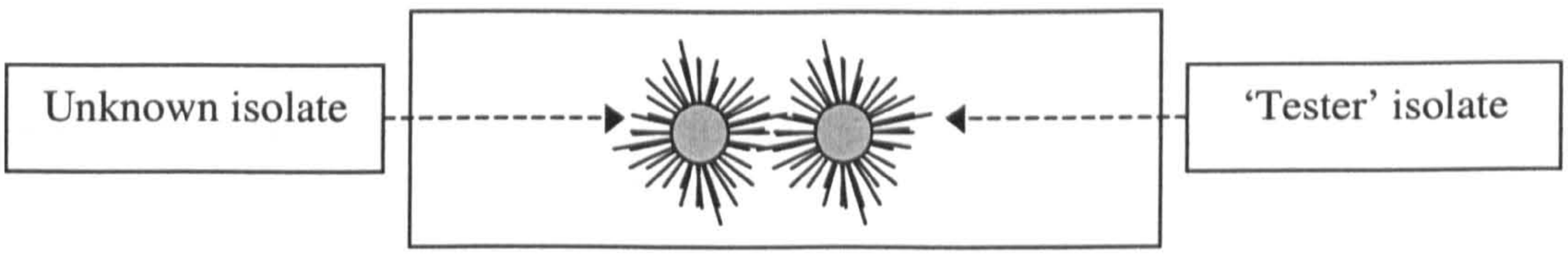


Figure 2.1 Clean slide method (Kronland & Staghellini, 1988) used to determine anastomosis group of *Rhizoctonia solani*

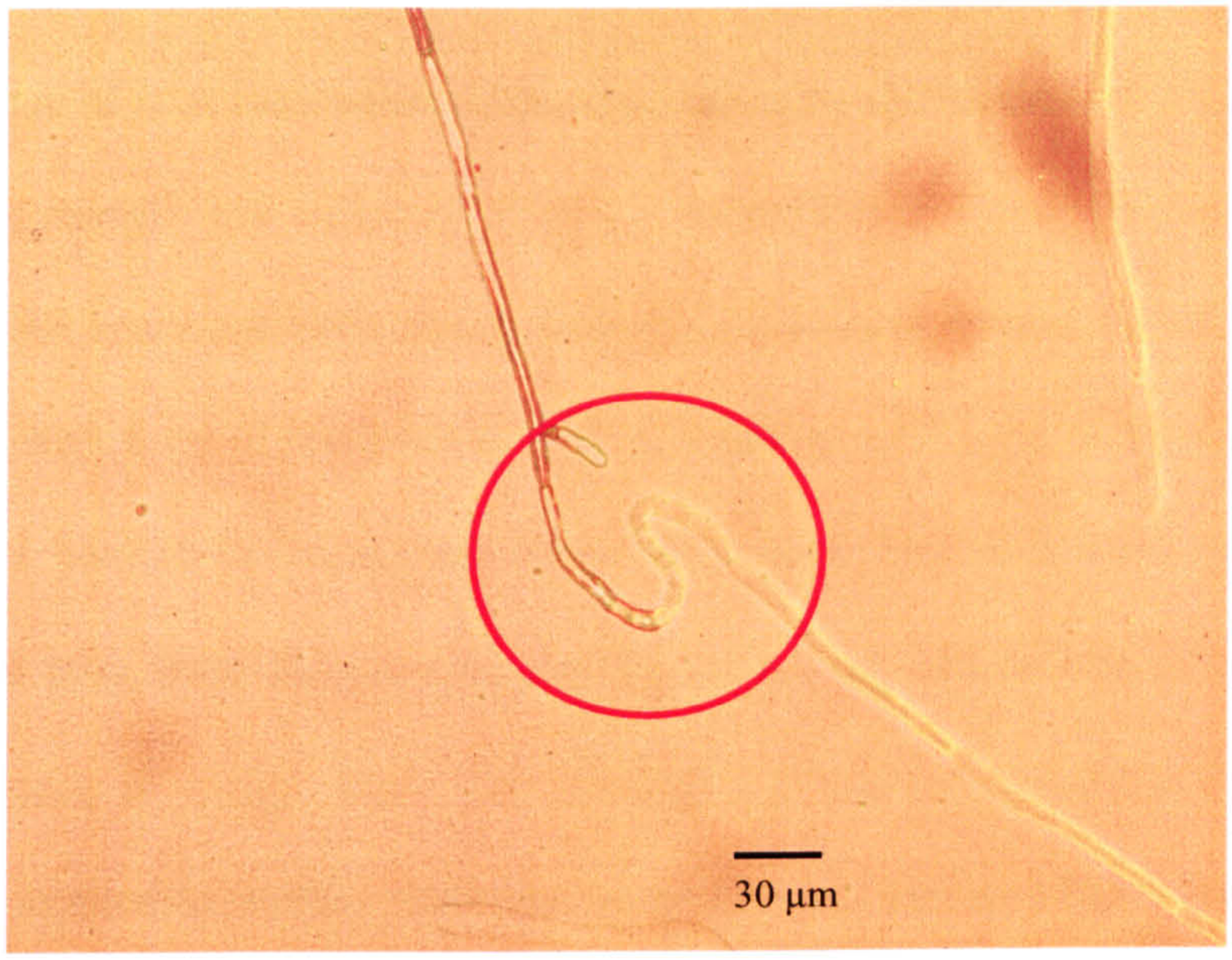


Plate 2.2 Hyphal anastomosis between two isolates of *Rhizoctonia solani* belonging to the same anastomosis group (Lietz DM-RB photo microscope)

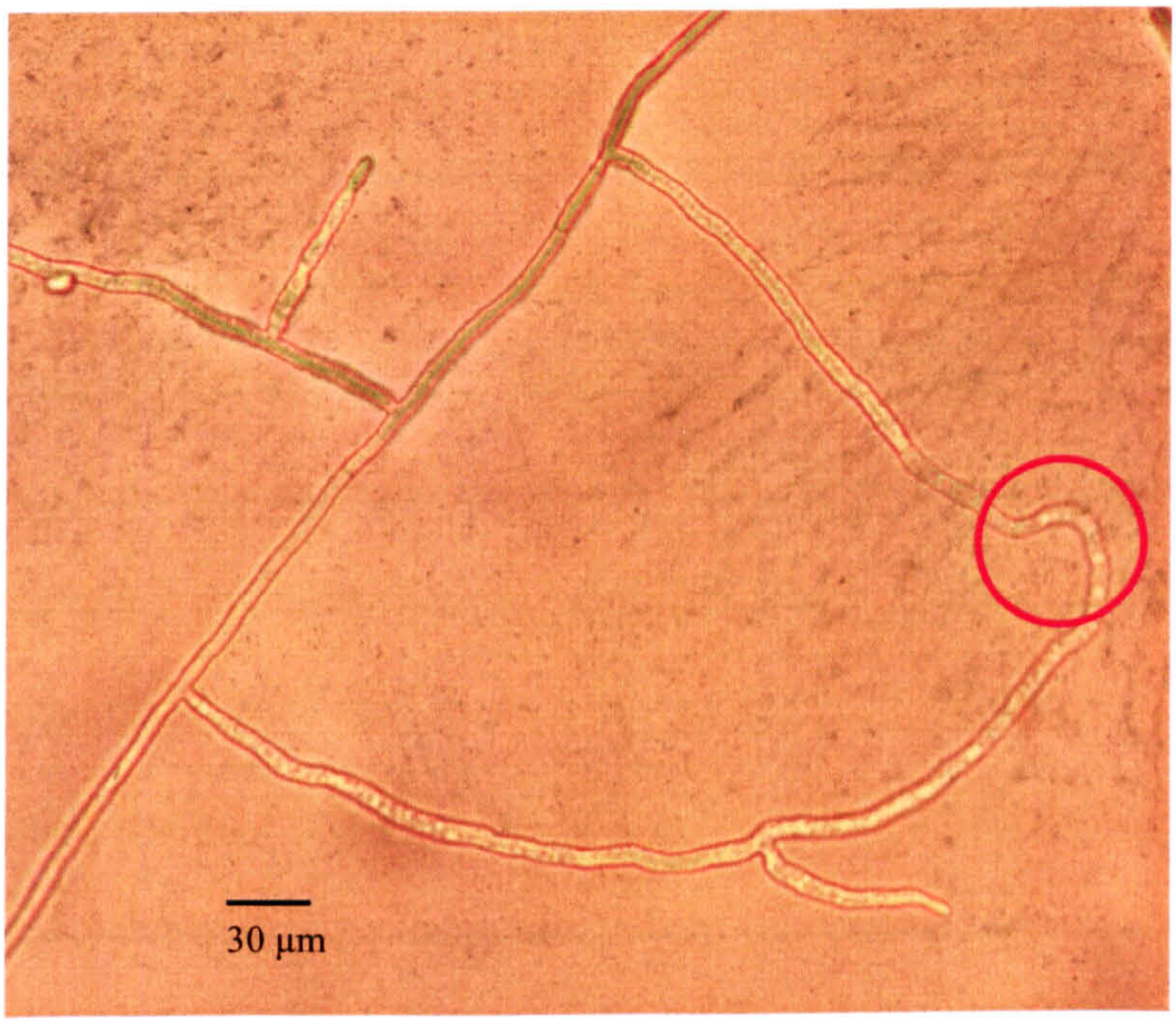


Plate 2.3 Self-anastomosis between hyphae of a single isolate of *Rhizoctonia solani* (Lietz DM-RB photo microscope)

2.1.3 Long term storage of R. solani *isolates*

Long term storage of *R. solani* isolates was achieved using the methods of Butler (1980). A sandy loam soil sample was collected from a field at Harper Adams University College, air-dried for ca.48 hours and then mixed with 4% (w/w) wheat bran. Screw cap vials (20 ml) were filled with 8 ml of the loam-bran mix, together with 3 ml of distilled water and autoclaved for 1 hour at 121°C on two consecutive days. The vials were then inoculated with a mycelial plug from the growing margin of a 7-day-old culture of *R. solani* and sealed with a bung of cotton wool. The soil/bran cultures were incubated at 23-27 °C where they are reputed to remain viable for up to 2 years (Butler, 1980). By sprinkling a few grains of the infested soil/bran mixture onto WA, it was possible to revive cultures.

2.1.4 Production and bulking of R. solani *inoculum*

For the purpose of inoculating experimental plants with *R. solani*, it was necessary to find a suitable method that would allow inoculum to be bulked up and applied easily. The methods of Papavizas & Davey (1962) were adapted and found to be successful for infecting potato plants with the pathogen. Whole crop maize was roughly milled before being mixed with silver sand and distilled water (20g maize: 980g sand: 200 ml distilled water) in a 1l conical flask and autoclaved for 60 minutes at 121°C. When the mixture had cooled, it was transferred to a polythene bag and inoculated with 6 plugs from the growing margin of a 7-day-old culture of *R. solani*. The inoculated bags of sand–maize media were sealed and incubated for 4-6 weeks at room temperature (ca.15°C). Following the incubation period, bags were examined for visible contaminants (*Penicillium* spp., *Rhizopus* spp.) and contaminated bags discarded. Before experimental use, the contents of inoculum bags were combined and homogenised in a cement mixer.

2.1.5 Assessment of R. solani *diseases*

For the purposes of assessing stem and stolon canker severity, two approaches were adopted; 1) the percentage of stolons infected and pruned were recorded 2) a key was devised to record the overall damage caused by stem and stolon infections (Table 2.1) on individual main stems. In 2001 the key was further modified to give greater partitioning of scores made on the proportion of secondary stems pruned and the proportion of stolons infected (Table 2.2).

The severity of black scurf was measured by estimating the percentage area occupied by sclerotia on washed tuber surfaces. This task was simplified by dividing the tuber into eight areas using a marker pen and by examining the assessment key used by James & McKenzie (1972).

2.2 Disease assessment of seed potato tubers

A disease assessment was undertaken on a sample of 50 seed tubers for each experiment to determine the incidence and severity of diseases on the tuber surfaces. This was particularly useful for establishing the amount of *R. solani* seed-borne inoculum present and for detecting any potentially damaging diseases such as powdery scab (*Spongospora subterranea* f. sp. *subterranea*) and skin spot (*Polyscytalum pustulans*)(Jeger *et al.*, 1996). Before assessment, the tuber samples were thoroughly washed to remove any soil and debris. Initially, a macroscopic assessment of surface diseases was made on the tubers following the methods described in section 2.1.5 for black scurf. The incidence of the pathogens *R. solani*, *Helminthosporium solani* (causal pathogen of silver scurf), *Colletotrichum coccodes* (causal pathogen of black dot) and *P. pustulans* occurring on the tuber eyes were determined by adapting the methods of Hide *et al.* (1968). Single eye

plugs were taken by inserting a No. 3 cork borer ca.15 mm deep into the rose end of tubers and breaking out the plug. The plug was then released from the cork borer using a dissecting needle and a scalpel was used to cut away the excess potato tissue to leave a 4-5 mm eye plug. Eye plugs were arranged on a 96 well micro-titre plate, which was placed in a sterile plastic box with 5 ml of sterile distilled water and incubated for 7 days at 15 °C. Following incubation, the eye plugs were examined with a binocular microscope (Magnification = x 30) and fungal pathogens were identified with the descriptions of Hide *et al.* (1968) and Domsch *et al.* (1993).

Table 2.1 Assessment key for stem canker (2000)

Score	Symptom descriptions
0	No symptoms occur on stem(s) (main and secondary) or stolons.
1	1-2 superficial lesions occur on stem(s) (main and secondary) or stolons. **Superficial lesions** appear as light brown blemishes. Affected tissue may appear slightly sunken.
2	More than 2 superficial lesions on stem(s) (main and secondary) or stolons. OR 1-2 necrotic lesions on stem(s) (main and secondary) or stolons. **Necrotic lesions** are deeper causing cracking and necrosis of the epidermis giving a corky appearance.
3	1-2 necrotic lesions cover the entire circumference of the main stem or secondary stem(s) but do not penetrate through the entire cross section of the infection site (**girdling**). OR more than 2 necrotic lesions occur on the stem(s) and stolons.
4	1-2 of the stolons are either **pruned** (the disease has advanced to such an extent that the vegetative body is severed) or completely necrotised (necrosis occurs throughout the entire cross section of infection site). OR more than 2 sites of girdling have occurred on the stem(s) (main and secondary) and stolons.
5	The main stem has been pruned or completely necrotised leaving a secondary stem (s) to predominate. OR more than 2 stolons are pruned.
6	The main stem has been pruned or completely necrotised leaving a secondary stem (s) to predominate. The secondary stem(s) has/have stem canker lesions (either superficial or necrotic)
7	The main stem and secondary stem(s) have been pruned or completely necrotised. Secondary stem(s) still exist above ground.
8	The stem is completely dead. No part of the stem exists above ground.

For multiple main stems, the following equation was used to calculate the stem canker severity index.

$$\frac{\text{Grand total of scores for main stems}}{\text{Number of main stems}} = \text{Stem canker severity score for plant}$$

Table 2.2 Assessment key for stem canker (2001)

Score	Symptom descriptions
0	No symptoms occur on stem(s) (main and secondary) or stolons.
1	1-2 superficial lesions occur on stem(s) (main and secondary) or stolons. **Superficial lesions** appear as light brown blemishes. Affected tissue may appear slightly sunken.
2	More than 2 superficial lesions on stem(s) (main and secondary) or stolons OR 1-2 necrotic lesions on stem(s) (main and secondary) or stolons. **Necrotic lesions** are deeper causing cracking and necrosis of the epidermis giving a corky appearance.
3	1-2 necrotic lesions cover the entire circumference of the main stem or secondary stem(s) but do not penetrate through the entire cross section of the infection site **(girdling)**. OR more than 2 necrotic lesions occur on the stem(s) (main and secondary). OR 10-25 % of the stolons are infected. Stolons either possess necrotic lesions or are **pruned** (the disease has advanced to such an extent that the vegetative body is severed) or **completely necrotised** (necrosis occurs throughout the entire cross section of infection site).
4	2 or more sites of girdling have occurred on the stem(s) (main and secondary). OR 25-50 % of the stolons are infected.
5	The main stem has been pruned or completely necrotised leaving a secondary stem (s) to predominate. OR 50-75 % of the stolons are infected.
6	The main stem has been pruned or completely necrotised leaving a secondary stem (s) to predominate. The secondary stem(s) has/have stem canker lesions (either superficial or necrotic) OR +75 % of the stolons are infected.
7	The main stem and ≥25-50% of the secondary stems have been pruned or completely necrotised.
8	The main stem and >50% of the secondary stems have been pruned or completely necrotised.
9	The stem is completely dead. No part of the stem exists above ground.

$$\frac{\text{Grand total of scores for main stems}}{\text{Number of main stems}} = \text{Stem canker severity score for plant}$$

2.3 Sampling and identification of potato cyst nematodes (PCN)

2.3.1 Sampling the soil for PCN

During field experiments, soil was sampled for initial and final population densities of PCN (Pi and Pf, respectively). In each experimental plot, 30 cores (10 x 1.5 cm) were taken by inserting an auger with a half-cylindrical blade to a depth of 15-20 cm. A W pattern sampling strategy was used in both field experiments. A recent study, investigating sampling patterns demonstrated that the 'W pattern' of sampling provided the most consistent and accurate results regardless of its orientation (Evans *et al.*, 2000). The soil (ca.1 kg) from each plot was transferred to cotton bags (235 x 395 mm) and was dried at 25 °C for 4-5 days in a drying room at Harper Adams University College.

2.3.2 Identification, quantification and species determination of PCN from soil

The number of cysts and eggs per gram of soil were estimated using the methods outlined by Southey (1970). Dried soil samples were sieved through a 4 mm gauge sieve and mixed thoroughly before a 200 g sub-sample was removed. The cysts from the sub-samples were extracted with a Fenwick can (Fenwick, 1940) taking care to avoid cross-contamination between samples. The resultant extractions were dried overnight. The following day, extractions were examined under a binocular microscope (magnification = x 20) and PCN cysts counted. Cysts belonging to other species of cyst nematode such as *Heterodera schachtii* were discarded. Fifty PCN cysts were removed from each sample and soaked in distilled water for ca.24 hours. The cysts were then crushed and rigorously mixed with 50 ml of distilled water. 1 ml aliquots of the cyst suspension were examined under a binocular microscope (magnification = x 40), where the number of eggs and juveniles were counted.

Using equation 1, the number of eggs g^{-1} soil could be calculated.

Eggs g^{-1} soil =

$$\left[\left[\frac{\text{Water (ml) in egg suspension}}{\text{No. cysts in egg count}}\right] \times \text{No. eggs ml}^{-1}\right] \times \left[\frac{\text{No. of cysts in cyst count}}{\text{Weight of soil used (i.e. 200 g)}}\right]$$

equation (1)

The ratio of *Globodera rostochiensis* and *G. pallida* species present in each soil sample was determined using polymerase chain reaction assays (PCR). The DNA of fifty cysts was homogenised, extracted, amplified and digested using the methods of Edwards *et al.*, (2001) and Dr. S. Edwards, Harper Adams University College (Pers. Com.). An example of a PCR gel is shown in Plate 2.4

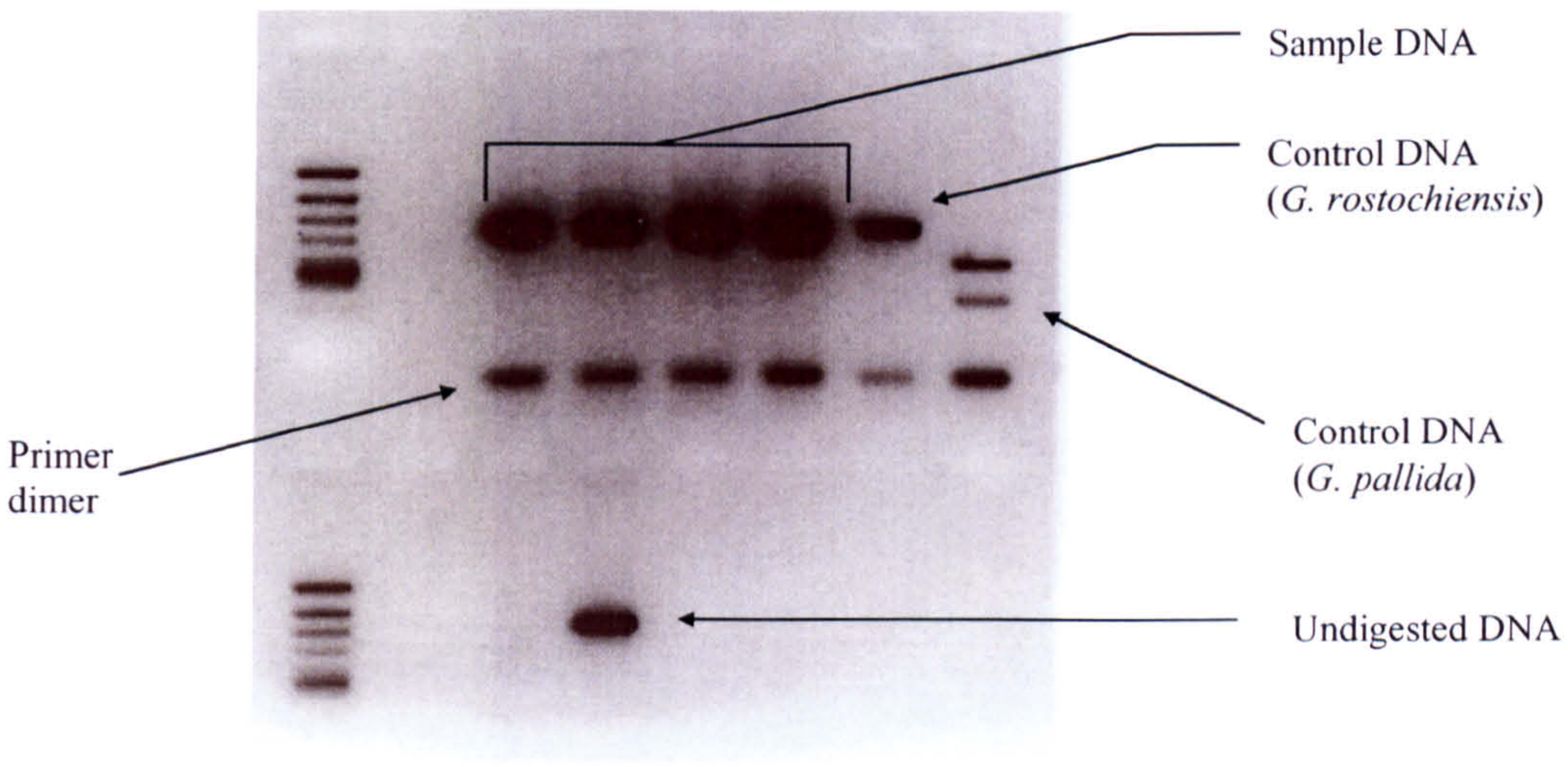


Plate 2.4 An agarose gel showing the amplification of *Globodera rostochiensis* and *G. pallida* digested DNA

2.3.3 Quantification of PCN invasion within potato roots

The density of juveniles within potato roots was determined using the methods described by Hooper (1986). For each experimental plant, roots were severed at their point of origin and cut into 2.5 mm pieces. Root pieces were mixed together before a 2 g sub-sample was removed and preserved in formic acetic alcohol (FAA). To enable the nematodes to be visualised, the root samples were stained with acid fuchscin. Stained samples were cut into 5 mm pieces and homogenised with 200 ml of distilled water using a laboratory blender. A 2 ml sub-sample of the suspension was transferred to a DeGrisse slide and the number of juvenile nematodes in each moult were counted using a binocular microscope (magnification = x 50). By combining the data from each moult, the total number of juveniles per g^{-1} root was calculated (equation 2).

$$\text{Juveniles } g^{-1} \text{ root} = \frac{\text{Total juveniles in 2 ml sub-sample} \times 100}{\text{Weight of root sample in g (2 g)}}$$

Equation (2)

CHAPTER 3.0 – GLASSHOUSE EXPERIMENTS

CHAPTER 3.0 GLASSHOUSE EXPERIMENTS

3.1 Introduction

Few studies have investigated relationships between the soil-borne fungus *Rhizoctonia solani* and the potato cyst nematode *Globodera rostochiensis* in their effects on potatoes. This is surprising considering that observations suggesting an association between the two organisms date back to the early part of the twentieth century (Morgan, 1925, 1926; Miles, 1930). While studies such as those of Grainger & Clark (1963) and Mazurkiewicz-Zapalowicz & Waker-Wójciuk (1994) have investigated interactions between *R. solani* and *G. rostochiensis*, these experiments have lacked detailed measurements of plant growth and disease severity. In addition, neither of these studies investigated the effect of variable densities of *G. rostochiensis* and *R. solani* on the potato plant.

The first glasshouse experiment (2000) described in this chapter utilises a similar experimental design to the studies of Grainger & Clark (1963) and Mazurkiewicz-Zapalowicz & Waker-Wójciuk (1994) and was initiated as a preliminary investigation of the interrelationships between *G. rostochiensis* and *R. solani*. The objective of this experiment was to investigate the independent and combined effects of *G. rostochiensis* and *R. solani* on the growth of the potato plant over two harvest dates, 4 and 6 weeks after planting.

Following the findings of field experiment 2000 (see Chapter 4), interrelationships between *G. rostochiensis* and *R. solani* were further explored in a second glasshouse experiment (2001). The objective of this experiment was to examine the effect of *R. solani* soil densities against variable population densities of *G. rostochiensis* on two potato cultivars. The null hypothesis for both glasshouse experiments was that *G. rostochiensis* infestations did not affect *R. solani* diseases of potato.

3.2 Materials and Methods

3.2.1 Glasshouse experiment 2000

In order to explore the independent and combined effects of *G. rostochiensis* and *R. solani* on potatoes, a simple randomised block experiment was designed. The treatments included two levels of *R. solani* (inoculated and uninoculated) and two levels of *G. rostochiensis* (infested and uninfested), which were assessed at four and six weeks after planting (see Table 3.1). Twenty replicates were used for each treatment.

Table 3.1 Experimental treatments used to examine the combined and independent effects of *G. rostochiensis* and *R. solani* on the growth of potato, the development of *R. solani* diseases and the infestation of potato roots by *G. rostochiensis*

Treatment number	*R. solani* inoculation	*G. rostochiensis* infestation
1	-	-
2	-	+
3	+	-
4	+	+

+ = *R. solani*/*G. rostochiensis* (12 eggs g^{-1} soil) present
- = *R. solani*/*G. rostochiensis* (12 eggs g^{-1} soil) absent

Prior to the experiment, a seed stock of the potato cultivar Maris Peer was obtained from Cambridge University Farm. Maris Peer was chosen for its susceptibility to *G. rostochiensis* (Anon, 2000) and its moderate susceptibility to *R. solani* (Dowley, 1972).

The potato seed was chitted for 3 weeks at ca.15°C, after which the sprout lengths measured ca.3-5 mm. A visual assessment of the tubers revealed that no black scurf was present.

Potato cyst nematode infested field soil (ca. 20 Kg) was collected from a selected site in Four-Gates Field at Harper Adams University College (Ordnance Survey Grid Reference: SJ 707195) and then dried at 25°C for 4-5 days. When dry, three 200 g sub-samples of soil were removed from the bulk of the material and the number of eggs per cyst determined using the methods described in Chapter 2 (section 2.3.2, page 60). A sample of 50 cysts was taken from the extracted float and the species determined using polymerase chain reaction (PCR). The results of the PCR revealed that the sample was pure *G. rostochiensis*. The number of cysts required for each *G. rostochiensis* treated pot was calculated using equation 3 where W is the weight of potting medium required to fill one experimental pot, P is the required initial population of *G. rostochiensis* and E is the mean egg count of *G. rostochiensis* from infested soil.

$$\frac{W \times P}{E} \qquad \text{equation (3)}$$

On the basis of *G. rostochiensis* economic damage thresholds (Brodie *et al.*, 1993) and processing limitations, 12 eggs g^{-1} of soil was used as an initial population density in pots with *G. rostochiensis* treatments. Using equation 3, it was calculated that 73 cysts would be required to infest a pot with 12 eggs g^{-1} soil of *G. rostochiensis*. Subsequently, 73 cysts were counted out into Eppendorf tubes for each experimental pot requiring *G. rostochiensis* infestation.

On 3 February 2000, the chitted potato tubers were planted with their rose ends facing upwards at a depth of 10 cm in John Innes No.2 ® sterilised loam based peat in 15 cm diameter pots. The potting medium of experimental pots to be infested with *G. rostochiensis* was thoroughly mixed with the pre-counted *G. rostochiensis* cysts from a single Eppendorf tube. *Rhizoctonia solani* inoculation was achieved by pouring a 25 ml

scoop of *R. solani* sand/maize meal inoculum (Chapter 2, page 55) around the tuber as it was planted. Pots receiving no *G. rostochiensis* or *R. solani* treatments served as controls.

Following planting, the pots were arranged in 10 randomised blocks on a bench in a glasshouse with a daytime temperature of 15°C, a night time temperature of 5°C and a 14 hour photo period. Watering was undertaken as required.

Two weeks after planting, measurements of plant emergence were taken. A plant was considered to have emerged if a shoot could be seen above the soil surface. For each experimental treatment the mean number of days taken for potatoes to emerge was calculated and termed the mean emergence time. Four weeks after planting, 10 replicate plants from treatments 1-4 were removed from their pots while the excess soil was carefully brushed away from the subterranean regions. Individual plants were placed in correspondingly labelled polythene bags and taken back to the laboratory where they were thoroughly washed under a gentle stream of flowing water to remove the remaining soil. Each plant was then assessed for stem canker symptoms (Chapter 2, pages 58-9) and the root systems were cut away, weighed and preserved for the determination of *G. rostochiensis* juvenile densities at a later date (Chapter 2, page 62). Stem and stolon numbers were recorded from the remaining plant material before the total haulm fresh weight (all plant material excluding the roots) was recorded. The plants were then packed into labelled linen bags and placed in an oven for 72 hours at 84°C after which, the contents of each linen bag was removed and immediately weighed. By subtracting the haulm dry weight from the fresh weight an estimation of percentage fresh weight was obtained. The same sequence of measurements was repeated on the plants harvested 6 weeks after planting.

3.2.2 Glasshouse experiment 2001

This experiment was designed to investigate variable densities of *G. rostochiensis* (four population densities) and *R. solani* (four inoculum densities) with two cultivars of potato. The treatments of the experiment are presented in Table 3.2 below: -

Table 3.2 Treatment structure of glasshouse experiment 2001, investigating variable densities of *G. rostochiensis* and *R. solani* on the growth of potato cultivars Charlotte and Estima and the development of *R. solani* diseases

Treatment number	Density of *G. rostochiensis* (eggs g^{-1} soil)	Quantity of *R. solani* inoculum (g)
1	0	0
2	0	15
3	0	30
4	0	60
5	10	0
6	10	15
7	10	30
8	10	60
9	20	0
10	20	15
11	20	30
12	20	60
13	30	0
14	30	15
15	30	30
16	30	60

The potato seed was chitted for 3 weeks at ca.15°C to produce 3-5 mm sprouts. A visual assessment of both the Charlotte and Estima seed revealed that no black scurf was present.

Potato cyst nematode infested soil was collected from a site known to have high populations of *G. rostochiensis* in Four-Gates Field at Harper Adams University College (Ordnance Survey Grid Reference: SJ 707195). Three 200 g sub-samples were removed from the bulk of the material and the number of eggs per cyst determined using the methods described in Chapter 2. A sample of 50 cysts was taken from the extracted float and assayed with the polymerase chain reaction (PCR) described in chapter 2 (page 61) to determine the species of PCN present. The results of the PCR revealed that the sample was pure *G. rostochiensis*. Using the egg count data and equation 3, the calculated number of *G. rostochiensis* cysts to infest 2 kilograms of John Innes No.2 ® at infestation densities of 10, 20 and 30 eggs g^{-1} soil was determined to be 299, 597 and 896 cysts, respectively.

An 8 kg stock of *R. solani* sand/maize-meal inoculum was prepared 4 weeks prior to the experiment. The number of colony forming units (cfu's) of *R. solani* within the sand/maize-meal inoculum was estimated, by adapting Warcup's soil plating method (Warcup, 1950). Small quantities (1 and 2 mg) of soil were distributed on low nutrient water agar (Agar No. 2, Lab M) amended with the antibacterial agents' streptomycin sulphate (50 mg l^{-1} agar) and chloramphenicol (50 mg l^{-1} agar). Before the sand/maize-meal inoculum was sampled, the total bulk of inoculum was mixed together in a large plastic drum. Eight samples (ca. 1 g) were taken from the inoculum bulk and transferred to sterile Petri dishes. Following a preliminary dilution series experiment on the *R. solani* inoculum, it was determined that 1 and 2 mg of *R. solani* inoculum would produce a measurable number of cfu's. Consequently, 1 and 2 mg sub-samples were taken from each of the samples and placed into Eppendorf tubes. Each tube was sprinkled evenly over a plate containing water agar, which was labelled accordingly. The plates were incubated at

15°C for 24 hours. Following incubation, the plates were examined under a binocular microscope (magnification x 30) and fungal colonies exhibiting typical *R. solani* diagnostic features were counted (Chapter 2, page 51). A sample of 10 colonies suspected to be *R. solani* were excised from the water agar plates and sub-cultured on potato dextrose agar where at least 95% were found to develop into the characteristic *R. solani* colonies. The results of the enumeration procedure are shown below in Table 3.3.

Table 3.3 An estimation of the colony forming units of *R. solani* existing within the sand/maize-meal inoculum prepared for glasshouse experiment 2001

Weight of *R. solani* sand/maize-meal inoculum (mg)	Mean number of *R. solani* colonies	*Rhizoctonia solani* cfu g^{-1} sand/maize-meal inoculum
1	61	61,000
2	135.25	69,625

On 28 March 2001, 160 pots (height: 23 cm, base diameter: 19 cm) were partially filled with an 870 g layer of 'John Innes No.2 ®' sterilised loam-based peat. A single potato tuber was planted into each pot by gently pressing the heel end of the tuber into the centre of the potting medium. This process was repeated 80 times for both cv. Charlotte and cv. Estima. Two kg of John Innes No.2 ® was mixed with the appropriate quantities of *G. rostochiensis* cysts and *R. solani* sand/maize-meal inoculum (Table 3.2) by vigorously shaking the constituents in a sealed polythene bag for 2 minutes. Following mixing, the treated potting medium was poured into a corresponding pot, which was labelled with the treatment details. An example of a treated experimental pot is shown in Figure 3.1. The total volume of potting medium used in each pot was 2.87 g. Five replicate pots were produced for each experimental treatment (Table 3.2).

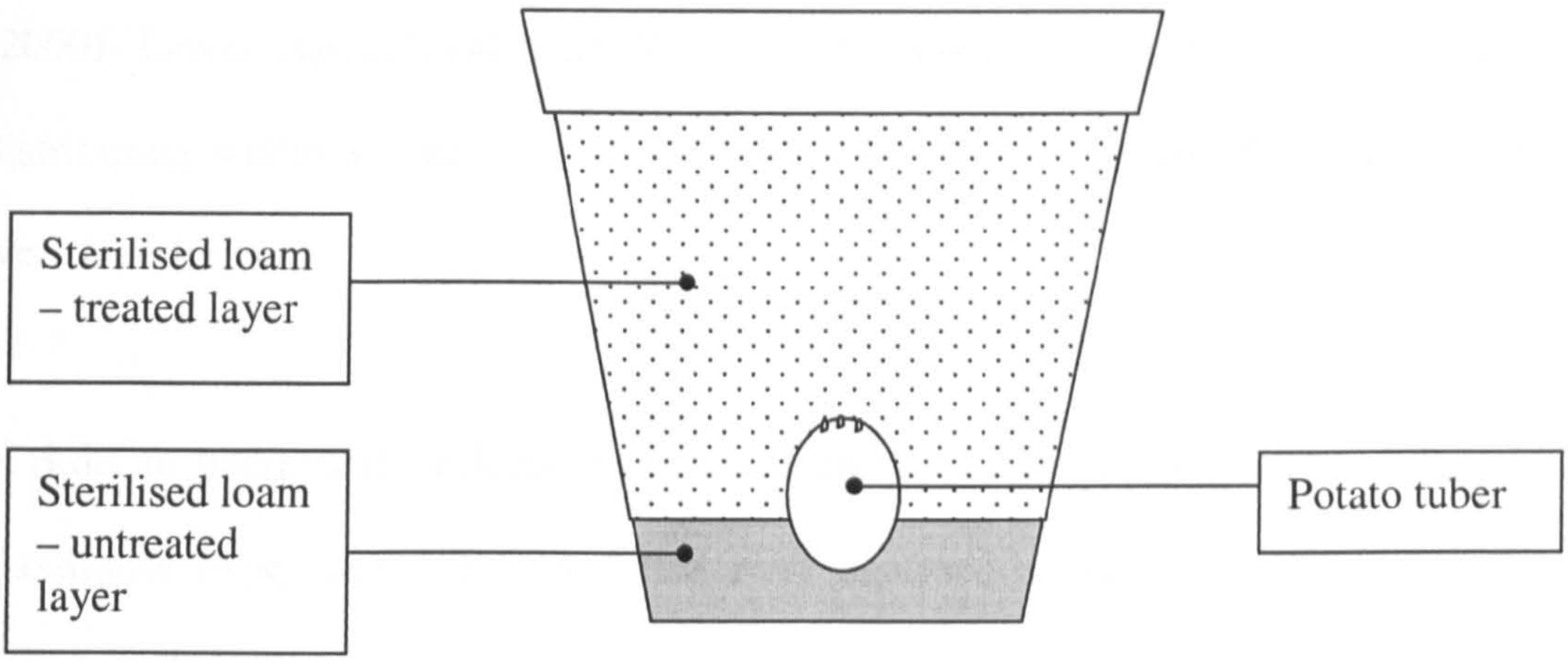


Figure 3.1 An example of an experimental pot used in glasshouse experiment 2001 showing the strata of treated and untreated John Innes No.2® potting medium

Following planting, the pots were arranged in 5 randomised blocks on two benches within a glasshouse with a daytime temperature of 15°C, a night time temperature of 5°C and a 14 hour photo period. Watering was undertaken as required. The percentage of emerged plants was determined on a daily basis up to 14 days after planting. Six weeks after planting, all plants were harvested and assessed using the procedures outlined for glasshouse experiment 2000.

3.2.3 Statistical analysis

All statistical analyses were conducted with the aid of Genstat – 5th edition, release 4.2 (2000), Lawes Agricultural Trust ©. Frequency histograms were used to examine the distribution within variates and data not showing a typical normal/Gaussian distribution were transformed.

In order to interpret the effects of different densities of *G. rostochiensis* and *R. solani* in glasshouse experiment 2001, the data were analysed using dose response-analysis of variance. This type of analysis indicates where linear and quadratic relationships exist between the explanatory dose rates and the response variate being examined, allowing multiple comparisons to be made.

3.3 Results

3.3.1 Glasshouse experiment 2000

3.3.1.1 Emergence

The daily emergence of potato plants under each experimental treatment is shown in Figure 3.2. Analysis of variance revealed a significant delay in emergence time (ca. 3-7 days) in both independent and combined treatments of *G. rostochiensis* and *R. solani* compared to the untreated plants (Table 3.4). However, least significant differences (L.S.D.'s) indicated no significant differences between plants either inoculated with *R. solani* alone or in combination with *G. rostochiensis* infestations.

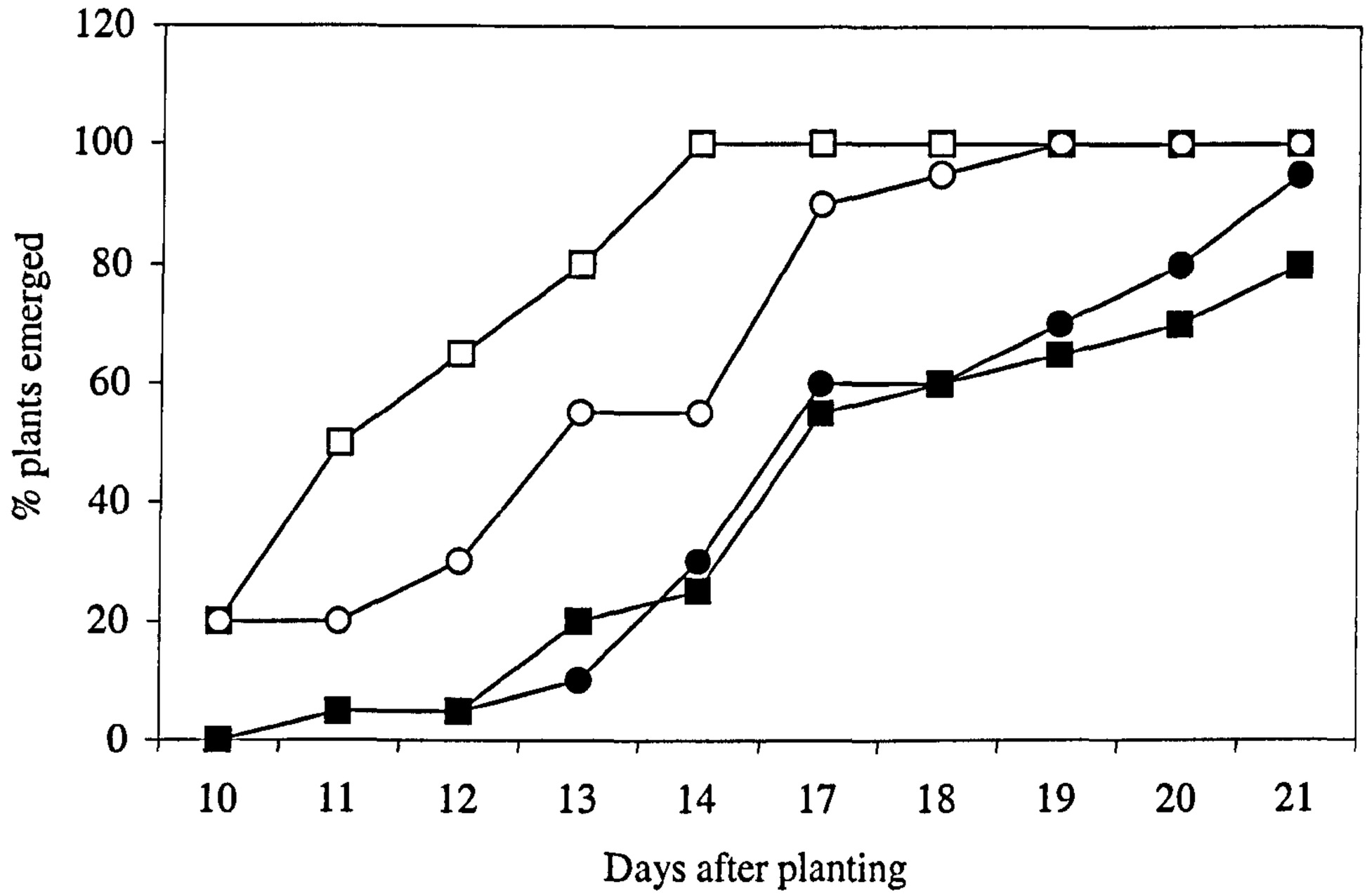


Figure 3.2 The percentage emergence of potato plants (cv. Maris Peer) in pots either left untreated (□), infested with *G. rostochiensis* (○), inoculated with *R. solani* (■), infested with *G. rostochiensis* and inoculated with *R. solani* (●), 10-21 days after planting glasshouse experiment 2000

Table 3.4 A summary of analysis of variance conducted on mean potato (cv. Maris Peer) emergence times (the time taken in days for plants to emerge) from experimental pots either left untreated, infested with *G. rostochiensis*, inoculated with *R. solani* or infested with *G. rostochiensis* and inoculated with *R. solani*

Treatments	Mean emergence time (days)*	S.E.M.	S.D.
Untreated	11.40a	0.432	1.930
Infested with *G. rostochiensis*	14.50b	0.742	3.317
Inoculated with *R. solani*	18.00c	0.880	3.934
Infested with *G. rostochiensis* Inoculated with *R. solani*	17.70c	0.778	3.481
Significance (P) = <0.001	d.f. = 67	L.S.D. (P=0.05) =1.980	%cv = 20.4

* Numbers followed by the same letter are not significantly different according to L.S.D. multiple range test

3.3.1.2 Four-week harvest

Table 3.5 shows the analysis of variance conducted on measurements obtained from plants harvested 4 weeks after planting. Measurements of stem and stolon canker infections caused by *R. solani* in the presence and absence of *G. rostochiensis* were analysed by using a nested analysis of variance. In general, mean *R. solani* infections appeared to be higher in plants inoculated with both *G. rostochiensis* and *R. solani* compared to plants inoculated with *R. solani* alone. However, these data sets were not found to be significantly different from one another, possibly due to the high variation within these measurements.

Comparing haulm fresh weights revealed significant differences between the experimental treatments ($P<0.001$). Least significant difference multiple range tests indicated a significant reduction in the haulm fresh weight of plants inoculated with *R. solani* compared to plants uninoculated with *R. solani*. However, plants either inoculated with *R. solani* alone or in combination with *G. rostochiensis* infestations did not have significantly different haulm fresh weights. No other significant differences were found between any of the other plant growth measurements taken.

Analysis of the total *G. rostochiensis* juveniles invading potato roots revealed no significant differences between pots infested with *G. rostochiensis* at planting. However, when individual juvenile stages were analysed, significantly higher numbers ($P<0.05$) of J2 juveniles were found in plants co-inoculated with *R. solani* compared to plants infested with *G. rostochiensis* alone. Insufficient J4-5 juveniles were observed to allow statistical analyses.

Table 3.5 The effect of infesting loam based John Innes No. 2® peat with *G. rostochiensis* (12 eggs g^{-1} soil), inoculating peat with *R. solani* inoculum (25ml) or combined infestation with *G. rostochiensis* and *R. solani* on (a) development of stem and stolon canker, (b) root invasion by *G. rostochiensis* juveniles and (c) potato plant development four weeks after planting potatoes (cv. Maris Peer)

(a)

Treatment	% stolons infected by *R. solani*	% stolons pruned by *R. solani*	Stem canker index
Untreated	-	-	-
G. rostochiensis only	-	-	-
R. solani only	28.1	24.4	4.8
G. rostochiensis and *R. solani*	39	31.1	5.3
d.f.	27	27	27
S.E.M. ($P<0.05$)	4.27	3.81	0.35
L.S.D. ($P=0.05$)	19.73	18.47	0.72
%cv	128.3	145.3	25.8

(b)

Treatment	Total *G. rostochiensis* juveniles g^{-1} root	J2 *G. rostochiensis* juveniles g^{-1} root	J3 *G. rostochiensis* juveniles g^{-1} root
Untreated	-	-	-
G. rostochiensis only	105	45	55
R. solani only	-	-	-
G. rostochiensis and *R. solani*	185	110	75
d.f.	27	27	27
S.E.M. ($P<0.05$)	17.26	11.39	9.05
L.S.D. ($P=0.05$)	68.4	50.16	40.75
%cv	101.0	141.1	136.6

(c)

Treatment	Haulm fresh weight *	Haulm dry weight	Root fresh weight	Total stems
Untreated	28.7a	2.087	10.21	1.90
G. rostochiensis only	22.6ab	1.549	8.21	1.70
R. solani only	20.8bc	1.561	8.42	2.10
G. rostochiensis and *R. solani*	15.6c	1.310	7.22	2.50
d.f.	27	27	27	27
S.E.M. ($P<0.05$)	1.57	0.11	0.45	0.27
L.S.D. ($P=0.05$)	7.42	0.58	2.40	1.66
%cv	36.9	38.7	30.7	88.1

* Numbers followed by the same letter are not significantly different according to L.S.D. multiple range test

3.3.1.3 Six-week harvest

Table 3.6 summarises the measurements recorded for plants harvested 6 weeks after planting. The results found were similar to those recorded during the four-week harvest. Plants inoculated with both *G. rostochiensis* and *R. solani* had a similar incidence and severity of *R. solani* diseases to plants inoculated with *R. solani* alone and were not statistically significantly different. Where *R. solani* inoculation was used, a significant reduction in root fresh weight ($P<0.001$), haulm fresh weight ($P<0.001$) and haulm dry weight ($P<0.001$) of plants was found.

As with the four-week harvest, infestation of potato roots by *G. rostochiensis* juveniles was significantly higher in plants co-inoculated with *R. solani* compared to plants infested with *G. rostochiensis* alone. Furthermore, *G. rostochiensis* juvenile stages 3 and 4 (J3-4) were also significantly higher in plants co-inoculated with *R. solani* compared to plants infested with *G. rostochiensis* alone. Due to the low number of juvenile stages J2 and J5 ANOVA could not be undertaken on these data.

Table 3.6 The effect of infesting loam based John Innes No. 2® peat with *G. rostochiensis* (12 eggs g^{-1} soil), inoculating peat with *R. solani* inoculum (25ml) or combined infestation with *G. rostochiensis* and *R. solani* on (a) development of stem and stolon canker, (b) root invasion by *G. rostochiensis* juveniles and (c) potato plant development six weeks after planting potatoes (cv. Maris Peer)

(a)

Treatment	% stolons infected by *R. solani*	Stem canker index
Untreated	-	-
G. rostochiensis only	-	-
R. solani only	45.8	5.9
G. rostochiensis and *R. solani*	45.6	5.7
d.f.	27	27
S.E.M. (P<0.05)	4.88	0.48
L.S.D. (P=0.05)	19.12	0.72
%cv	91.2	26.9

(b)

Treatment	Total *G. rostochiensis* juveniles g^{-1} root	J3 *G. rostochiensis* juveniles g^{-1} root	J4 *G. rostochiensis* juveniles g^{-1} root
Untreated	-	-	-
G. rostochiensis only	115	5	90
R. solani only	-	-	-
G. rostochiensis and *R. solani*	270	50	170
d.f.	27	27	27
S.E.M. (P<0.05)	22.03	5.93	15.0
L.S.D. (P=0.05)	79.20	29.52	59.60
%cv	89.7	233.9	100.0

(c)

Treatment	Haulm fresh weight *	Haulm dry weight *	Root fresh weight *	Total stems
Untreated	84.8a	7.11a	17.72a	3
G. rostochiensis only	79.2ab	6.55b	16.09a	2
R. solani only	62.5bc	4.95c	13.22b	4
G. rostochiensis and *R. solani*	63.9c	4.83c	11.78b	2.4
d.f.	27	27	27	27
S.E.M. (P<0.05)	2.48	0.26	0.58	0.30
L.S.D. (P<0.05)	10.22	1.18	2.85	1.67
%cv	15.3	21.9	21.1	61.8

* Numbers followed by the same letter are not significantly different according to L.S.D. multiple range test

3.3.2 Glasshouse experiment 2001

3.3.2.1 Emergence

A summary of the dose response-analysis of variance undertaken on the emergence of potato plants (cv. Charlotte and cv. Estima) is presented in Table 3.7. Increasing the dose of *R. solani* inoculum significantly increased the time taken for plants to emerge. More importantly, results from the analysis of variance revealed a significant effect of the co-inoculation of *G. rostochiensis* and *R. solani* on emergence. Plate 3.1 and Figures 3.3 and 3.4 illustrate the longer emergence times in experimental pots inoculated with 60 g of *R. solani* sand/maize-meal inoculum and infested with 10, 20 or 30 eggs g^{-1} soil of *G. rostochiensis* compared to pots uninfested with *G. rostochiensis* for both the potato cultivars Charlotte and Estima.

Table 3.7 Dose response-analysis of variance on the time (days) taken for potato plants (cv. Charlotte and cv. Estima) to emerge in pots treated with a range of *G. rostochiensis* and *R. solani* densities in glasshouse experiment 2001

Source of variation	Significance (P)	L.S.D. ($P = 0.05$)	S.E.M
G. rostochiensis	0.298	1.031	0.521
R. solani	<0.001	1.458	0.521
Potato cultivar	<0.005	1.458	0.368
G. rostochiensis * *R. solani*	<0.05	2.976	1.042
d.f. = 124	%cv = 20.2		

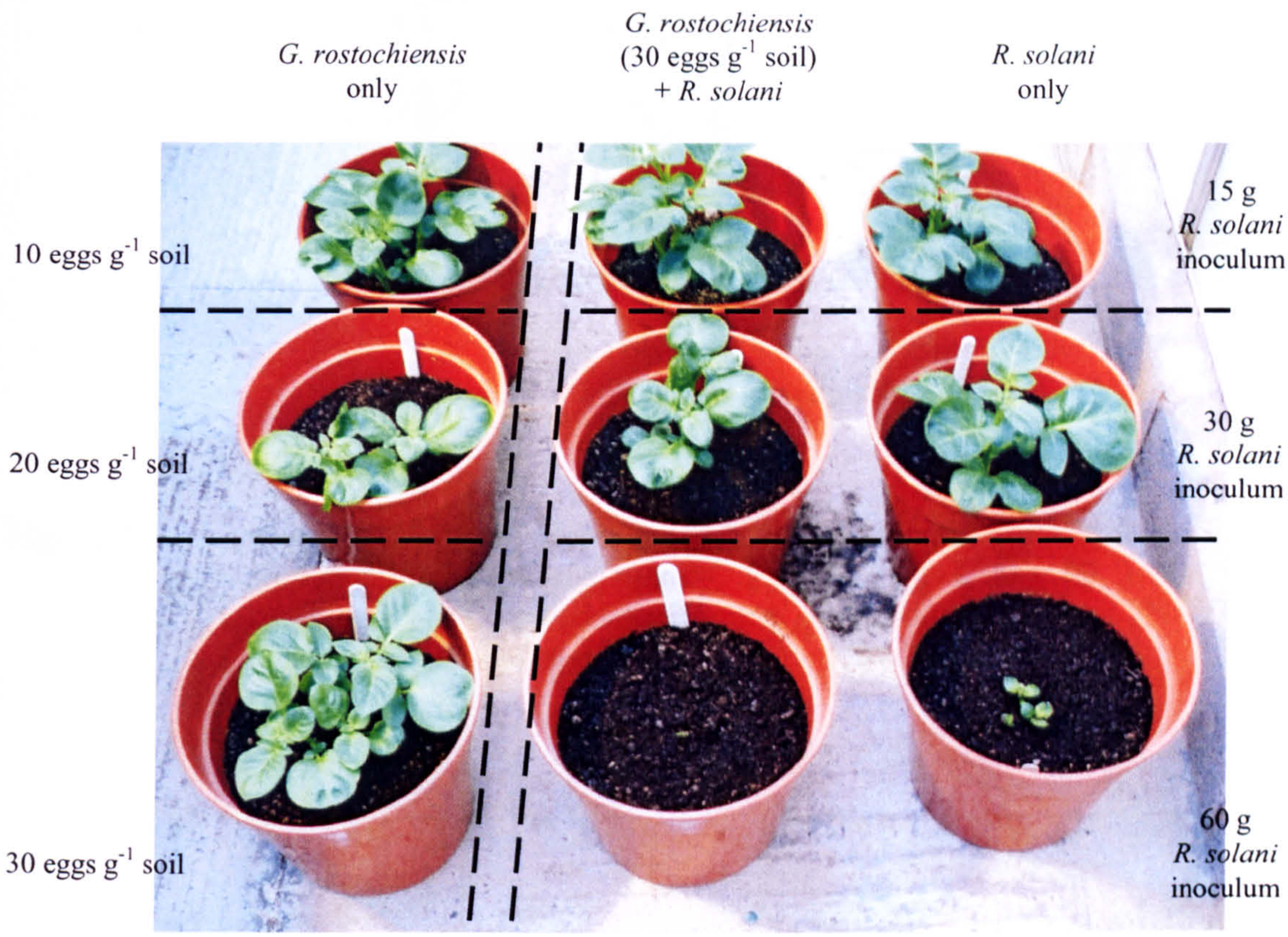


Plate 3.1 Emergence of potato plants (cv. Estima) in potting medium infested with either 10, 20 or 30 eggs g^{-1} soil of *G. rostochiensis* (left column), infested with 30 eggs g^{-1} soil of *G. rostochiensis* and inoculated with 15, 30 or 60 g of *R. solani* sand/maize-meal inoculum (centre column) or inoculated with 15, 30 or 60 g of *R. solani* sand/maize-meal inoculum and uninfested with *G. rostochiensis* (right column), four weeks after planting glasshouse experiment 2001

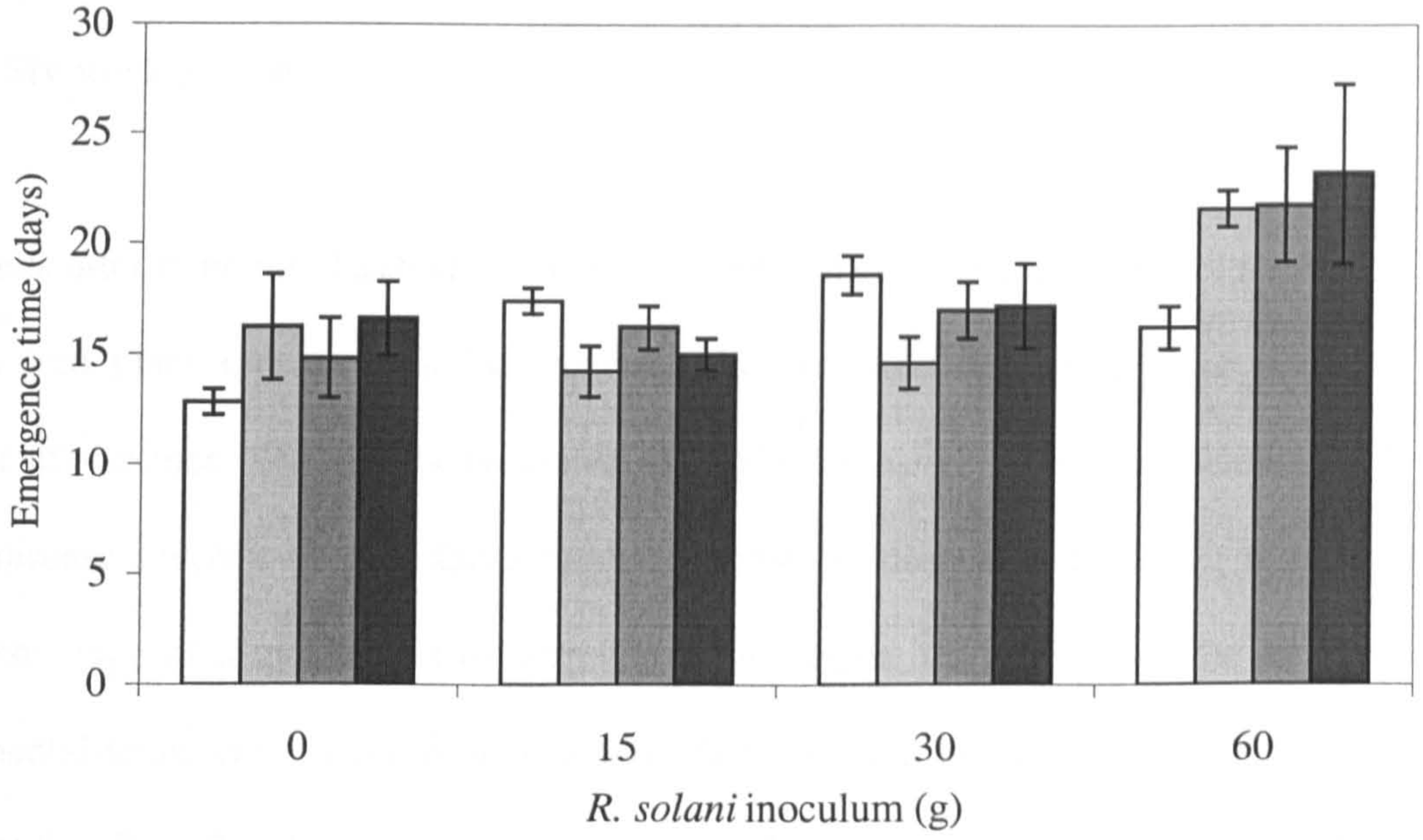


Figure 3.3 Emergence of potato plants (cv. Charlotte) in potting medium inoculated with *R. solani* and infested with 0 (□), 10 (▒), 20 (▓), 30 (■) eggs g^{-1} soil of *G. rostochiensis* during glasshouse experiment 2001. Error bars show the standard error of the mean

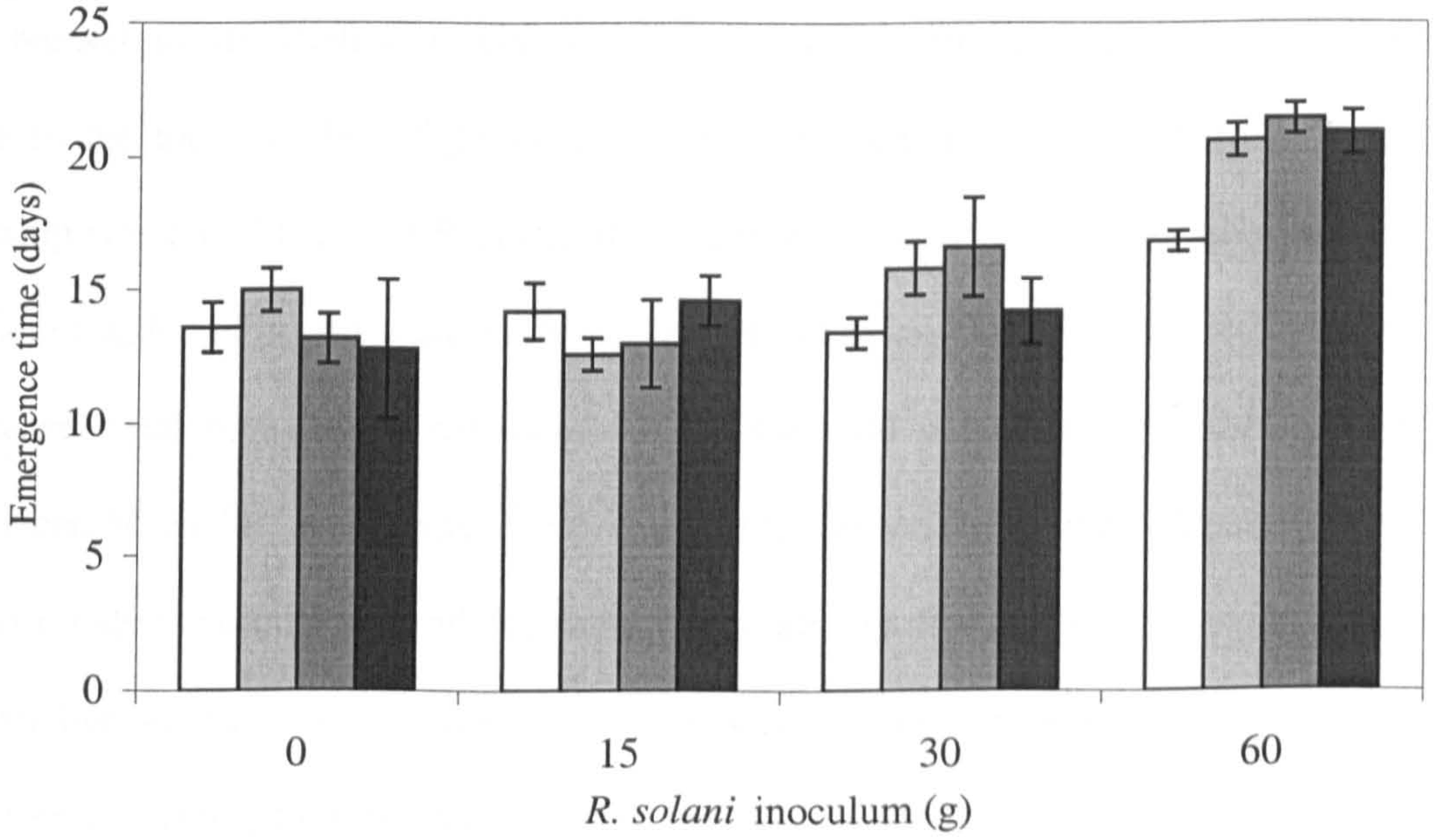


Figure 3.4 Emergence of potato plants (cv. Estima) in potting medium inoculated with *R. solani* and infested with 0 (□), 10 (▒), 20 (▓), 30 (■) eggs g^{-1} soil of *G. rostochiensis* during glasshouse experiment 2001. Error bars show the standard error of the mean

3.3.2.2 Six-week harvest

Six weeks after planting glasshouse experiment 2001, measurements of *R. solani* disease severity and plant growth from harvested plants were analysed using dose response-analysis of variance. The results presented in Table 3.8 clearly show an increase in *R. solani* disease severity in accordance to the incremental dosage of *R. solani* inoculum. Whilst this type of response was expected, it helps support the efficacy of the *R. solani* experimental treatments. *Globodera rostochiensis* population densities were not found to significantly affect *R. solani* diseases. However, by observing the treatment means for both cultivars, there were indications that *G. rostochiensis* densities had an effect on *R. solani* stem canker in the potato cv. Estima but not the cv. Charlotte.

Table 3.9 shows the result of dose response-analysis of variance undertaken on plant growth measurements. Both *R. solani* and *G. rostochiensis* densities caused a significant increase in the total number of potato stems. Furthermore, a significant positive linear relationship was found between *R. solani* inoculum and *G. rostochiensis* densities and the subsequent number of potato stems in cv. Estima but not in cv. Charlotte. Figure 3.5 shows the positive trend between *G. rostochiensis* densities and potato stem numbers of cv. Estima when 60 g of *R. solani* was incorporated within experimental pots. Measurements of stolon number, haulm fresh and dry weight were all significantly reduced by *R. solani* treatments but not by *G. rostochiensis* densities. Consequently there were no significant effects from interacting treatments of *R. solani* and *G. rostochiensis*.

Analysis of variance revealed that *R. solani* treatments had no significant effect on the number of juvenile *G. rostochiensis* invading potato roots.

Table 3.8 Dose response-analysis of variance showing the effect of *R. solani* inoculation, *G. rostochiensis* infestation and cultivar treatments on the development of *R. solani* diseases on potatoes (cv. Charlotte and cv. Estima), 6 weeks after planting glasshouse experiment 2001

Response Variate	Explanatory variates	Significance (*P*)	L.S.D. (*P* = 0.05)	S.E.M
Stem canker index	*R. solani* inoculation	<0.001	0.5271	0.1883
	G. rostochiensis infestation	0.063	0.5271	0.1883
	R. solani inoculation * *G. rostochiensis* infestation	0.892	1.0541	0.3766
	Potato cultivar	0.357	0.3727	0.1331
		d.f. = 124	%cv =	30.6
% stolons infected	*R. solani* inoculation	<0.001	4.14	11.58
	G. rostochiensis infestation	0.847	4.14	11.58
	R. solani inoculation * *G. rostochiensis* infestation	0.403	8.28	23.17
	Potato cultivar	0.895	2.93	8.19
		d.f. = 124	%cv =	80.6

Table 3.9 Dose response-analysis of variance showing the effect of *R. solani* inoculation, *G. rostochiensis* infestation and cultivar treatments on measurements of plant growth on potatoes (cv. Charlotte and cv. Estima), 6 weeks after planting glasshouse experiment 2001

Response Variate	Explanatory variates	Significance (*P*)	L.S.D. (*P* =0.05)	S.E.M
Total stems	*R. solani* inoculation	<0.001	0.838	0.299
	G. rostochiensis infestation	<0.05	0.838	0.299
	R. solani inoculation * *G. rostochiensis* infestation	0.259	1.676	0.599
		<0.025 lin †		
	Potato cultivar	0.002	0.592	0.212
		d.f.=124	%cv =	52.2
Total stolons	*R. solani* inoculation	<0.001	2.434	0.870
	G. rostochiensis infestation	0.434	2.434	0.870
	R. solani inoculation * *G. rostochiensis* infestation	0.423	4.869	1.739
	Potato cultivar	0.763	1.721	0.615
		d.f. = 124	%cv =	39.7
Haulm fresh weight	*R. solani* inoculation	<0.001	13.02	4.65
	G. rostochiensis infestation	0.837	13.02	4.65
	R. solani inoculation * *G. rostochiensis* infestation	0.531	26.04	9.30
	Potato cultivar	<0.001	9.21	3.29
		d.f. =123	%cv =	31.5
Haulm dry weight	*R. solani* inoculation	<0.001	1.365	0.487
	G. rostochiensis infestation	0.367	1.365	0.487
	R. solani inoculation * *G. rostochiensis* infestation	0.345	2.729	0.975
	Potato cultivar	<0.001	0.965	0.345
		d.f. =123	%cv =	43

† = Indicates a linear relationship

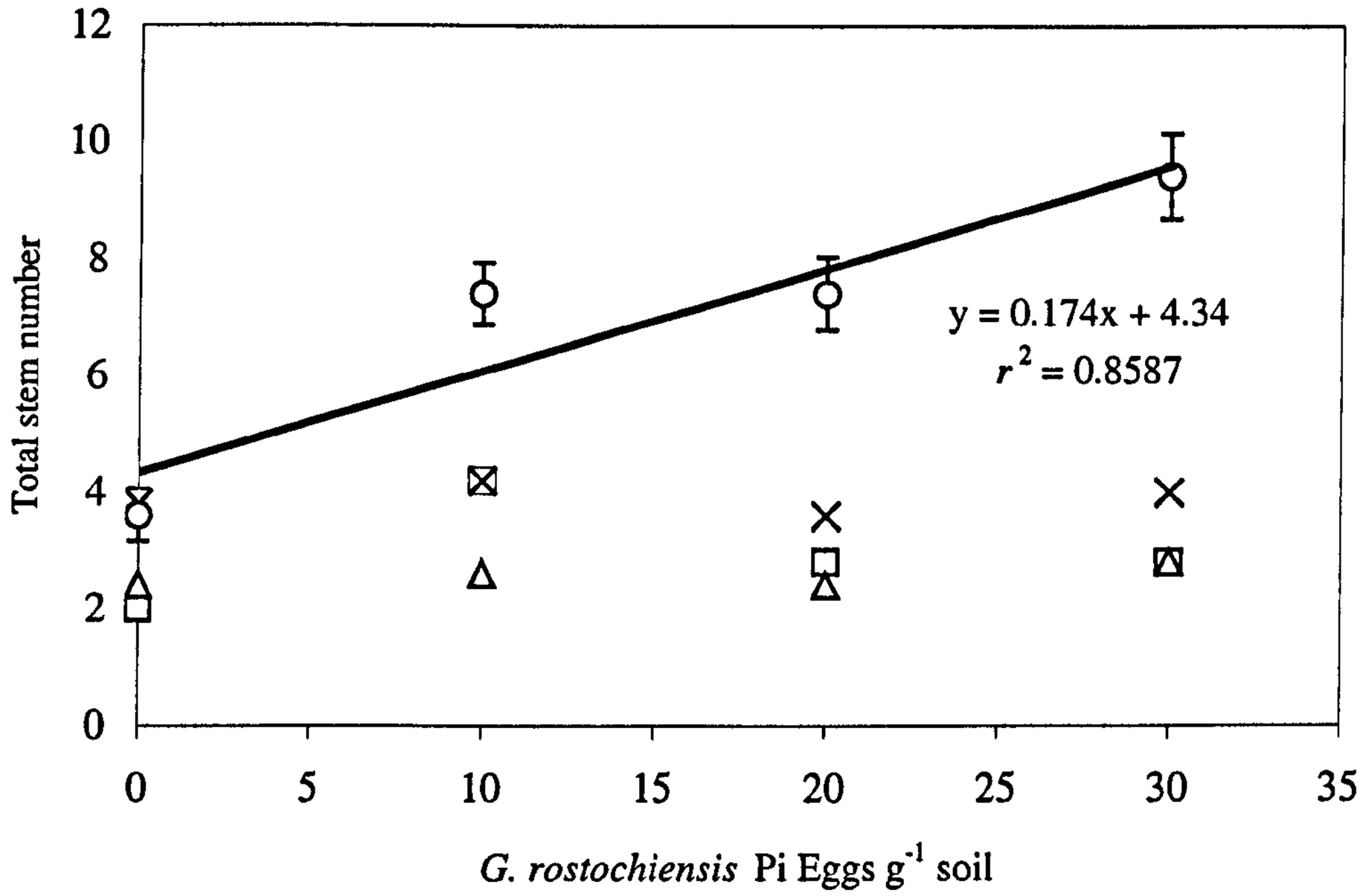


Figure 3.5 The relationship between soil densities of *G. rostochiensis* and the total number of potato stems (cv. Estima) in pots treated with 60 g of *R. solani* maize-meal/sand inoculum (○), 30 g of *R. solani* inoculum (X), 15 g of *R. solani* inoculum (Δ) and 0 g of *R. solani* inoculum (□) six weeks after planting glasshouse experiment 2001. Error bars denote standard error of the mean

3.4 Discussion

Both glasshouse experiments have given some indication of a synergistic interaction between *G. rostochiensis* and *R. solani*, although a significant increase in *R. solani* diseases in response to *G. rostochiensis* infestations was not observed in either of the two studies. In 2001, the length of time for cv. Charlotte and cv. Estima to emerge was significantly increased when plants were exposed to the highest dose rate of *R. solani* inoculum (60 g) and infestations of *G. rostochiensis* compared to plants treated with the lower dose rates of *R. solani* inoculum (15 and 30 g) with or without *G. rostochiensis* infestations. Since *R. solani* infections are known to delay the emergence of potatoes (Banville, 1989; Hide *et al.*, 1992), it could be hypothesised that early *R. solani* infections of developing shoots were increased in pots that were additionally infested with *G. rostochiensis*. Further glasshouse experiments, which examine *R. solani* disease progression at earlier harvest dates, could be used to explore this hypothesis.

Six weeks after planting glasshouse experiment 2001, a significant positive linear relationship was found between the number of secondary stems of cv. Estima and soil densities of *G. rostochiensis* in pots treated with the highest dose rate of *R. solani* inoculum (60 g). The fact that no such relationships were found with lower dose rates of *R. solani* inoculum or pots uninoculated with *R. solani*, further supports the hypothesis for a synergistic interaction between *G. rostochiensis* and *R. solani*. One possible explanation could be that *G. rostochiensis* infestations increased the severity of *R. solani* stem-based disease. Typical symptoms of *R. solani* damage on potato include the pruning of shoots and stems, resulting in an increase in the production of secondary stems (Hide *et al.*, 1992). Whilst no significant differences in disease symptoms were found in 2001, increased stem canker severity was observed in the meaned data for the cv. Estima but not cv. Charlotte. Further work using cv. Estima with the higher dose rate of *R. solani* inoculum and higher

numbers of replicate treatments could provide a clearer picture than the results found within this experiment.

In previous glasshouse studies, the combined presence of *G. rostochiensis* and *R. solani* has been found to significantly suppress the growth of potato (Grainger & Clark, 1963; Dunn & Hughes, 1964; 1967; Mazurkiewicz-Zapalowicz & Waker-Wójciuk, 1994). For example, Mazurkiewicz-Zapalowicz & Waker-Wójciuk (1994) observed significantly higher reductions in the sprout length of potatoes (cv. Mila) co-inoculated with *G. rostochiensis* and *R. solani* compared to potatoes inoculated with either organism alone. In addition, Grainger & Clark (1963) observed that potato tuber yields were approximately 35% lower when potatoes (cv. Epicure) were co-inoculated with *G. rostochiensis* (0.9 cysts g^{-1} soil) and *R. solani* in comparison to potatoes inoculated with either organism alone or left uninoculated. In contrast, potato growth measurements such as haulm fresh weight did not significantly differ between combined and independent treatments of *G. rostochiensis* and *R. solani* in the present glasshouse experiments. This may reflect the differences in *G. rostochiensis* infestation levels used by other workers. For example, Grainger & Clark (1963) used 0.9 cysts g^{-1} soil, which approximately equates to 180-450 eggs g^{-1} soil according to Evans & Stone (1977). Consequently, the comparably lower *G. rostochiensis* infestation rates used in the current experiments may have been too low to produce the effects seen by other authors. Furthermore, there are also other experimental differences such as potato cultivar, method of *R. solani* inoculation and density of *R. solani* inoculum that may have contributed to the contrasting results.

Both glasshouse experiments showed no indication of antagonism between *G. rostochiensis* and *R. solani*. These findings contradict those of Janowicz *et al.* (1994) who observed reduced numbers of *G. rostochiensis* juveniles, eggs and cysts on potatoes co-inoculated with *R. solani*.

Results from glasshouse experiment 2000 indicated that higher numbers of *G. rostochiensis* juveniles would invade the roots of plants co-inoculated with *R. solani* compared to plants infested with *G. rostochiensis* alone at both 4 and 6-week harvests. Associations between fungal infection of plants and increased numbers of plant parasitic nematodes have been reported (Vrain, 1987; Zahid *et al.*, 2002). Increased nematode densities in plants infected with fungal pathogens have previously been attributed to the activity of fungal enzymes (Nordmeyer & Sikora, 1983), fungal modifications to the rhizosphere such as increased production of CO_2 (Edmunds & Mai 1966ab) and the disruption of plant resistance mechanisms to nematodes (Hasan, 1985). In the current work, further investigations would need to be undertaken to support these findings, especially since *G. rostochiensis* juvenile densities were not increased in the 2001 glasshouse experiment.

Although results from both experiments have suggested that interactions occur between *G. rostochiensis* and *R. solani*, there is still a requirement for further evidence to support this hypothesis, especially since no direct relationship was observed between *G. rostochiensis* infestation and the development of *R. solani* diseases. One limitation of the glasshouse work was the difficulty in obtaining higher population densities of *G. rostochiensis* due to the impractical numbers of cysts that would require manual counting. Higher population densities than those used may have provided a more profound treatment effect than those seen in these experiments. Grainger & Clark (1963) achieved higher population densities of *G. rostochiensis* by using infested field soil with a pre-determined population density. However, there is no certainty that field soil will be free of soil-borne *R. solani* inoculum. The problem of obtaining higher *G. rostochiensis* soil densities could be overcome by reducing the size of the experimental pots and using cured potato seed pieces such as cores taken from the rose end of the tubers. This type of approach would reduce the quantity of potting medium that would require infesting with *G. rostochiensis* allowing more replicates

and treatments to be undertaken. Experiments using treatments with a wider range of population densities could provide greater scope to undertake alternative methods of analysis such as linear regression.

CHAPTER 4.0 – FIELD EXPERIMENTS

CHAPTER 4.0 FIELD EXPERIMENTS

4.1 Introduction

During 2000 and 2001, field experiments were conducted on two sites at Harper Adams University College to investigate the impact of concomitant populations of *Globodera rostochiensis* and *Rhizoctonia solani* on the growth and yield of potatoes. As previously discussed, disease complexes involving plant parasitic nematodes and soil-borne fungi are less frequently examined under natural conditions (Chapter 1). Moreover, the literature reviewed during this work indicates that interactions between *G. rostochiensis* and *R. solani* have not yet been investigated under field conditions. The requirement for more field experimentation in the study of disease complexes is also recognised by a number of other workers (Wallace, 1978; Swarup, 1990; Abawi & Chen, 1998; Hillocks, 2001). Crops grown in field experiments reach their natural maturity, which means that assessments of produce quantity and quality are comparable to that of a commercial crop. Samples and measurements taken throughout the growing season of the crop may also provide more detailed information on the temporal occurrence of the interaction between *G. rostochiensis* and *R. solani* and its effect on particular developmental stages of the crop. In addition, many authors have drawn attention to the potentially influential biotic and abiotic factors that are excluded in studies investigating disease complexes under controlled environments (Wallace, 1989; Swarup, 1990).

The objectives of this work were to (i) determine whether a relationship exists between potato cyst nematode infestation and the development of *R. solani* diseases under field conditions and (ii) evaluate the effect of any such interactions on the development of the potato crop. The null hypothesis for both field experiments was that *G. rostochiensis* infestations did not affect *R. solani* diseases on potatoes.

4.2 Materials and methods

4.2.1 Field experiment 2000

Four-Gates Field at Harper Adams University College, Newport, Shropshire, UK (Ordnance Survey Grid Reference: SJ 707195) was selected for its highly variable potato cyst nematode (PCN) population densities. During 1999, the site was used for a field experiment that explored the efficiency of integrating various PCN management practices (including treatments of nematicides, fumigants and resistant potato cultivars) in reducing PCN densities (Minnis, 2001). This array of treatments subsequently produced a mosaic of PCN population densities in a very small area.

During April 2000, the experimental area was sub-soiled, ploughed and planting beds were formed. Beds were divided into two ridges, which allowed a single line of planting to be undertaken in each. Before planting, forty experimental plots (7.32 m wide, 5 m long) consisting of two beds each (four ridges) were marked out and sampled for PCN initial population densities (Pi) (see Chapter 2). Cyst samples (50 cysts) were taken from each Pi sample and speciated using polymerase chain reaction (PCR). The results indicated that plots contained pure *G. rostochiensis* populations. Plots having similar *G. rostochiensis* Pi's were paired and randomly appointed to be either *R. solani* inoculated or uninoculated to produce two inoculum treatments.

The potato cultivar Désirée was chosen for its low resistance to *R. solani* (Little *et al.*, 1988), its susceptibility to *G. rostochiensis* (Anon., 2000) and its tolerance to drought (Anon., 2000). Drought tolerance was desirable because the experiment was to be positioned within a

commercial wheat field, where irrigation would not be possible. Upon obtaining the potato seed (Class VTSC 1, Seed size 35-45) the number of tubers required (3200 + 300 spare) were arranged in single layers within chitting trays with their 'rose' (apex, where the majority of eyes/sprouts are clustered) ends facing upwards. The seed was then incubated at 15°C±5 in continuous light for ca. 2 weeks. Following this 'chitting' period, the sprouts had developed root primordia and measured ca.15 mm in length (Plate 4.1). A disease assessment of 100 tubers taken at random revealed that 2% had black scurf present. In addition, a further microscopic assessment of 20 tubers under a binocular microscope (magnification = x 20) revealed that no mycelium of *R. solani* was present.

The experiment was initiated on the 16 May 2000. Tubers were planted to a depth of 15 cm at 25 cm intervals using a hand held potato planter (Plate 4.2), which was developed specifically for this task. A length of twine equal to that of the plot, marked with 25 cm graduations was used to gauge the planting spaces. Two rows of 20 tubers were planted in each bed to make a plot containing four rows. The inner two rows of the plot (harvest rows) were either inoculated with sand-maize meal inoculum (Chapter 2, page 55) infested with *R. solani* (inoculated plots) or with sterilised sand (uninoculated plots). Inoculation was undertaken by adding a 50 ml scoop of homogenised inoculum or sterilised sand with each tuber being planted (Plate 4.2). The outer two rows of the plots were not inoculated and served as guard (discard) rows to protect against edge effects.

The soil type of Four Gates Field was classified as a sandy loam using soil maps from the Bridgnorth series (Beard, 1988). The pH of each experimental plot was determined using standard methods (Anon, 1986) and is detailed in Appendix 4. Measurements of soil temperature were taken every 30 minutes using 2 Tiny Talk® data loggers (Gemini data

loggers UK Ltd, Chichester, West Sussex, UK) that were buried at a depth of 15 cm at 2 different locations within the experiment (see Appendix 2). Rainfall was recorded from the weather station at Harper Adams University College and is presented in the Appendix 2.

During the early stages of the crop (0-4 weeks), recordings of plant emergence were taken from the harvest rows of each plot on a daily basis. Plants were considered to have emerged if the developing shoot was found above the soil surface. Later, three weeks after planting, weekly assessments of canopy development were taken where the percentage area occupied by four plants was measured with the use of a ground cover grid (Burstall & Harris, 1983) fitted over the harvest rows.

Plate 4.1 Désirée potato seed used in field experiment 2000

Plate 4.2 Inoculating potatoes (cv. Désirée) with a scoop of sand/maize meal medium infested with *Rhizoctonia solani*

At 4, 6 and 8 weeks after planting, 4 plants (2 from each harvest row) were harvested from each plot, taking care to avoid damaging their roots and stolons. The first 2 plants in each row were not harvested and 2 plants were left between each harvest to reduce edge effects. Plants collected from each plot were placed in labelled polythene bags along with ca.10ml of distilled water to prevent them from wilting. Following harvesting, the plants were washed, placed in clean polythene bags and moved to a cold store where they were maintained at 4°C.

Before any assessments were undertaken, the plants were tagged with a reference label. Symptoms of stem and stolon canker were assessed using several methods. Firstly, *R. solani* diseases were measured with the aid of a stem canker severity key (Chapter 2, page 58). The incidence of stolons showing canker lesions and the incidence of pruned stolons were also recorded.

Plant development was examined by taking measurements of stem numbers (main and secondary), stolon number, tuber number (>5 mm), tuber weight and haulm fresh weight. On completion of the assessments, the haulm (excluding tubers and roots) was weighed, packed into labelled linen bags and placed in an oven for 72 hours at 84°C. After this period, the contents of each linen bag were removed and immediately weighed. By subtracting the haulm dry weight from the fresh weight an estimation of percentage fresh weight was obtained.

The densities of *G. rostochiensis* juveniles within the roots of each plant were determined using the methods described in Chapter 2 (section 2.3.3, page 62). Since part of this process involved removing the entire root system, root fresh weight was measured.

Eighteen weeks after planting (19 September 2000), the entire experiment was desiccated using diquat ('Reglone®', 200g l^{-1}) at 4 l ha^{-1} and was left for a period of ca.2-3 weeks to allow the skins of the tubers to set. After this period, the tubers from 8 plants were harvested from each plot (4 from each harvest row) using a fork. The harvested area of each plot was sampled for final population densities of PCN (Pf) and these were taken back to the laboratory for drying.

The harvested tubers (>5mm) were washed carefully and assessed for black scurf incidence and severity. The tubers were then weighed and graded into size fractions (<45mm, 45-65mm, 65-85mm, >85mm).

4.2.2 Field experiment 2001

The results obtained from the field experiment in 2000 gave an indication that *G. rostochiensis* and *R. solani* were interlinked in a damaging disease complex of potatoes. Consequently, a further field experiment to examine this relationship was deemed necessary. As mentioned previously, field experiment 2000 was conducted on a site, which had variable population densities of *G. rostochiensis* as a result of a range of artificial (experimental) treatments that had been applied during the previous year. In contrast, the second field experiment was conducted at Swans Leasow Field, Harper Adams University College, Newport, Shropshire, UK (Ordnance Survey Grid Reference: SJ 715198) which had not been exposed to such treatments. Whilst the population densities of PCN were less variable (0-108 eggs g^{-1} soil) than those in 2000 (5-220 eggs g^{-1} soil), the spatial characteristics of the nematode densities were more typical of those found to occur in commercially used fields (Riding & Parker, 2000).

For continuity between experiments, the cultivar 'Désirée' (class SE2, seed size 35-55) was obtained and chitted under the same light and temperature regimes used in the first field experiment. A sample of 100 tubers was assessed for surface diseases and the presence of pathogens was determined using a microscopic eye-plug test (see Table 4.1) (Hide *et al.*, 1968)

Table 4.1 Disease assessment of potato seed (cv. Désirée) used in 2001 field experiment showing a) the % incidence and severity of skin diseases and b) the % incidence of pathogens on excised eyes

a)

	Disease Severity				
Disease	1%	>1-5%	>5-12.5%	>12.5%	Incidence
Black scurf	0	1	1	0	2
Powdery scab	0	0	0	0	0
Common scab	18	44	5	2	69
Skin spot	0	0	0	0	0
Black dot	1	0	0	0	1
Silver scurf	7	17	5	3	32
Other	0	0	0	0	0

b)

Pathogen	% incidence
Rhizoctonia solani	3
Helminthosporium solani	3
Colletotrichum coccodes	0
Polyscytalum pustulans	0

The results of the disease assessment revealed a low incidence of both black scurf and the causal pathogen *R. solani*. Although a high incidence of silver scurf (*Helminthosporium solani*) and common scab (*Streptomyces scabies*) was found on the tubers, these diseases are generally regarded to be non-suppressive to the growth and yield of the potato (Agrios, 1988; Errampalli *et al.*, 2001).

Initially, the approximate position of the experiment was sited by sampling the entire field for PCN densities with a 20 m grid (Figure 4.1). At each intersect of the grid a point sample was taken using a trowel to remove ca.1 kg soil (0-15 cm depth). Once PCN population densities had been estimated, an area in the southeastern corner of the field was selected due to the range of Pi's found. This area appeared to be partially occupied by a 'focus' of the PCN population (Been & Schomaker, 1996; Boag *et al.*, 2000). Further sampling of PCN populations was undertaken with a 5 m grid (40 x 20 m overall), to provide more detailed information on the selected area. The corners of the sampling area were mapped with digital geographical positioning systems (DGPS) (Sokkia ® Hawkeye DGPS, Datum House Electra Way, Crewe Business Park, Crewe, Cheshire, CW1 6ZT, UK) so that the experimental area could be easily located during subsequent work. The results of the 5 m-grid point sampling (Figure 4.2) were used to decide upon the positioning of the experimental plots.

Following bed formation, a selected area within the 5 m-grid (Figure 4.2) was divided into 12 blocks of 6 plots (see Figure 4.3) and sampled for PCN initial population densities. In addition, PCR assays were undertaken on a sample of 50 cysts taken from each plot in order to determine the proportions of species present. Two pairs of plots were selected from each block on the basis of having similar PCN densities. Paired plots were randomly assigned treatments of either *R. solani* inoculation or sterile sand (uninoculated). Although the remaining

(unpaired) plots did not form part of the experiment they were still planted but marked as 'Discard'. Figure 4.3 shows a plan of the experiment.

The soil type of Swans Leasow Field was classified as loamy sand by using standard methods to determine sand, silt and clay fractions (Anon, 1986). The pH of each experimental plot was determined using standard methods (Anon, 1986) and is detailed in Appendix 5. Measurements of soil temperature were taken every 80 minutes using 3 Tiny Talk® data loggers that were buried at a depth of 15 cm at 3 different locations within the experiment (see Appendix 3). Rainfall was recorded from the weather station at Harper Adams University College and is presented in Appendix 3.

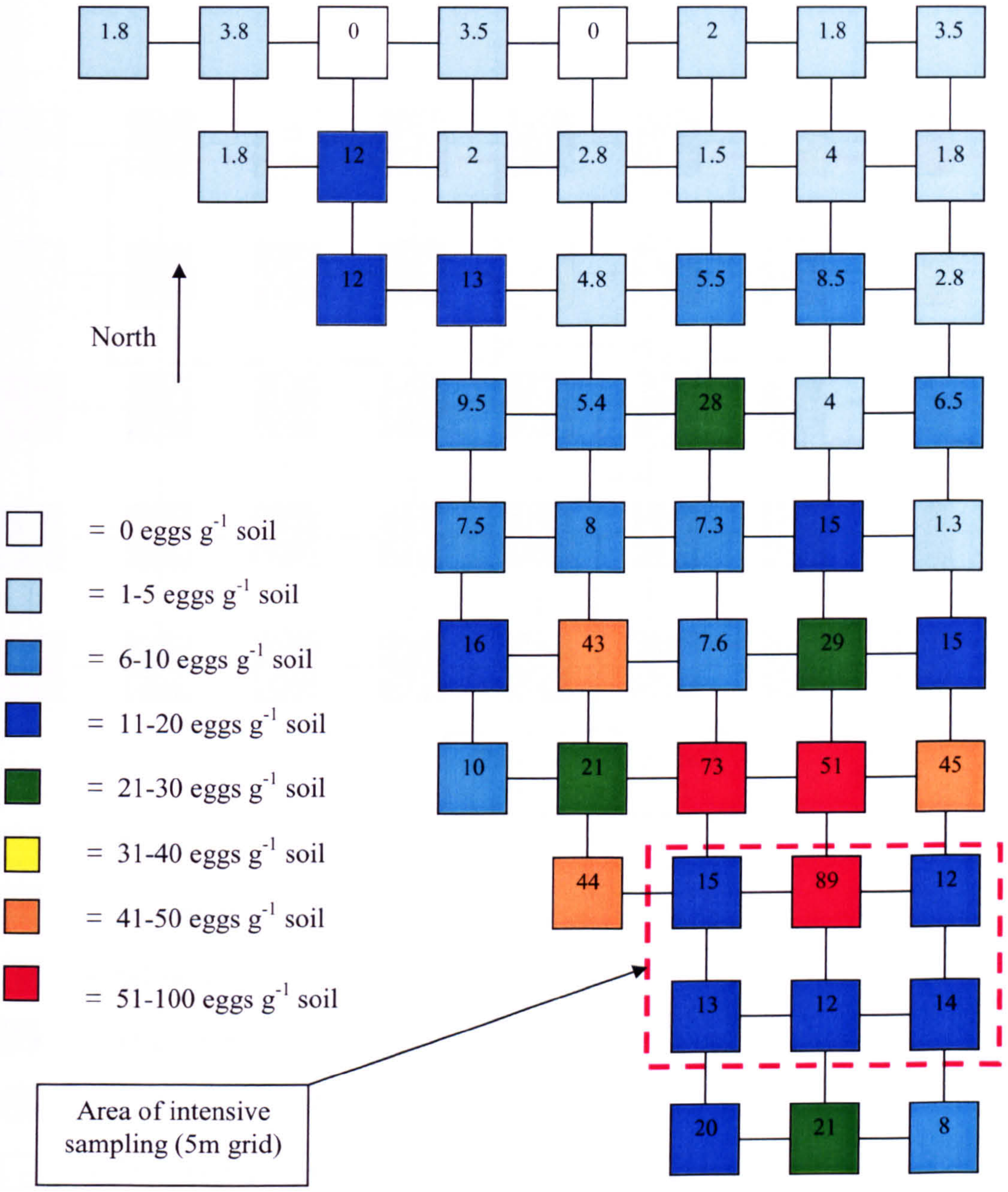


Figure 4.1 Initial population densities of potato cyst nematode (Pi) recorded in Swan's Leasow Field (Harper Adams University College) on a 20m-sampling grid in 2001

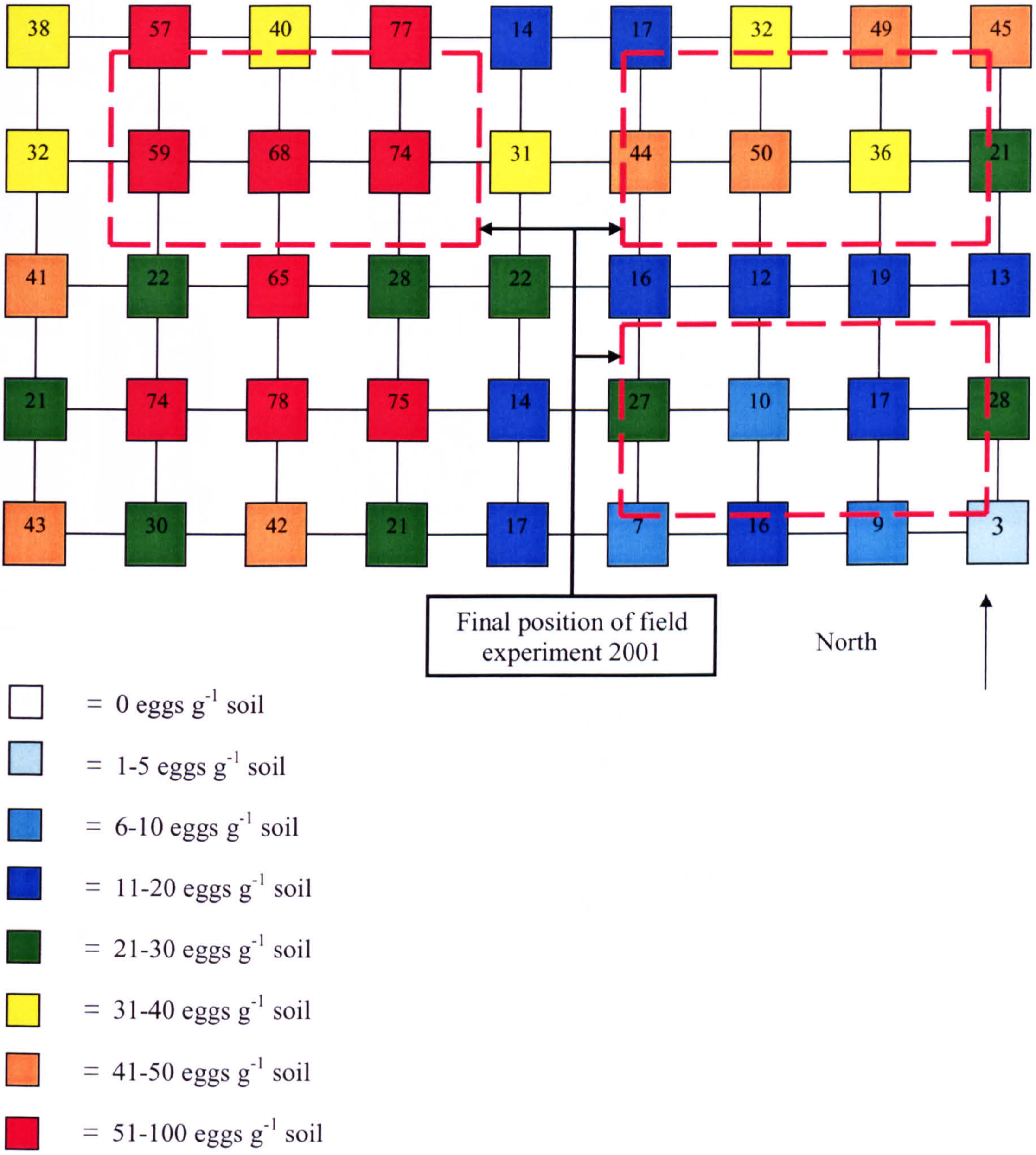


Figure 4.2 Intensive 5m sampling undertaken in Swans Leasow Field, Harper Adams University College showing the potato cyst nematode densities (eggs g^{-1} soil) within the experimental area for field experiment 2001

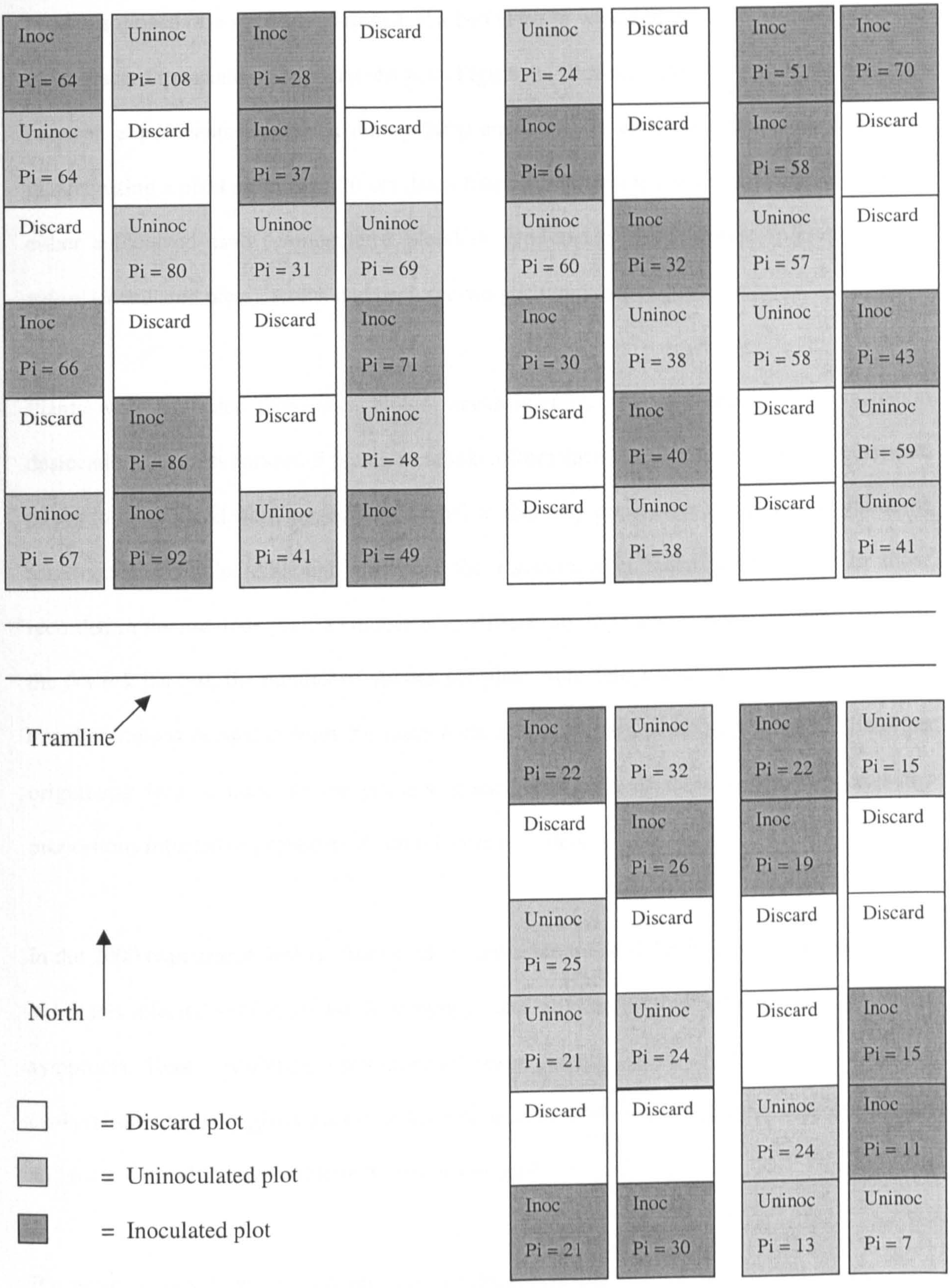


Figure 4.3 Plan of field experiment 2001 showing PCN initial population densities per plot

Plots comprised of a single, non-ridged, flat bed (1.86 m wide and 3.2 m long) which had two outer discard rows and a single harvest row. Figure 4.4 illustrates the changes in plot structure between experiments conducted during 2000 and 2001. In each row, 15 tubers were hand planted using a plant spacing of 20 cm. Each tuber planted in the harvest row received 50 g of either autoclaved sand (uninoculated plots) or sand-maize meal medium infested with *R. solani* (inoculated plots), which had been pre-weighed into disposable cups.

Plants were harvested from each plot 6 weeks after planting and again following haulm desiccation. At each harvest, 5 plants were taken from each plot. Prior to the 6-week harvest, plants to be sampled were tagged with a label so that they could be recognised throughout the assessment schedule. Although many of the measurements taken were similar to those recorded in the previous year, a number of modifications and new assessments were made. At the 6-week harvest, the number of stolons per plant were categorised into 'primary', 'lateral' (smaller stolons occurring from the same node as the primary stolon) or 'branched' (stolons originating from a node on the primary stolon). For each of these stolon categories the proportions infected or pruned by *R. solani* were recorded.

In the 2000 experiment, lesions (root canker) and sclerotia were found on the roots of 6-week old plants infected with *R. solani*. A scoring system was designed to individually assess these symptoms. Roots exhibiting no symptoms were given a score of 0, roots bearing 1-5 cankers/sclerotia were given a score of 1, roots bearing 5-10 cankers/sclerotia were scored as 2 and roots with >10 cankers/sclerotia were scored as 3.

By using a 'box type' grader, the tubers sampled in the second harvest (18 weeks after planting) were graded by size into 10 mm fractions; i.e. 10-20, 20-30 etc. up to grade >70mm.

Tubers in each size fraction was counted, weighed and assessed for black scurf by examining each tuber for the percentage area infected and the number of sclerotia present.

It was hoped that a smaller and more compact plot structure would help to reduce the variation of PCN densities and thus produce a clearer picture on their interaction with *R. solani*. In order to investigate the significance of the interaction between *G. rostochiensis* and *R. solani* on the development of black scurf, the number of harvests taken in the earlier stages of the crop were reduced to a single harvest at 6 weeks. Consequently, the shorter distance between the plants assessed for stem canker infections and those assessed for black scurf was more likely to be subject to similar PCN infestations making comparisons between these assessments more feasible.

4.2.3 Statistical analysis

All statistical analyses were conducted using Genstat – 5th edition, release 4.2 (2000), Lawes Agricultural Trust ©. Frequency histograms were used to examine the distribution within variates and data not showing a typical normal/Gaussian distribution were transformed.

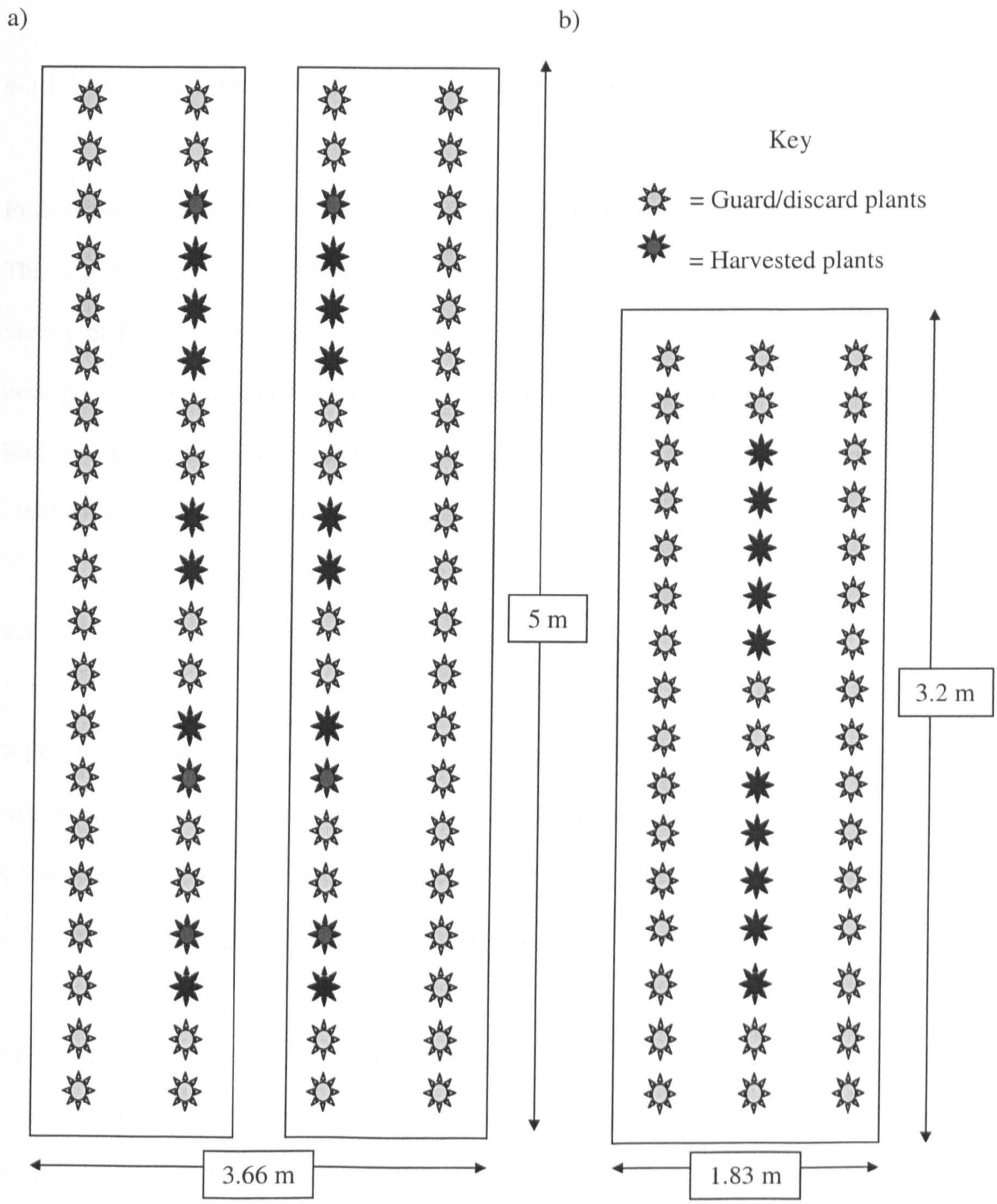


Figure 4.4 Plot designs used in field experiment a) 2000 and b) 2001

4.3 Results

4.3.1 Field experiment 2000

4.3.1.1 Potato cyst nematode initial population densities (Pi)

Pi densities were found to be highly variable (Table 4.2) ranging from 5 to 221 eggs g^{-1} soil. This was expected due to the fact that 'Four-Gates' Field had previously been exposed to a variety of PCN management practices during 1999. Furthermore, the fact that potatoes had been grown in the field in two consecutive years preceding this work would have undoubtedly lead to some of the high populations recorded. PCR assays revealed that populations were >95% *G. rostochiensis* and 0 % *G. pallida*.

4.3.1.2 Emergence and canopy development

Whilst undertaking daily assessments of emergence, it was apparent that tubers treated with *R. solani* inoculum were far slower to emerge than those untreated (Plates 4.3 and 4.4). Figure 4.5 shows the mean percentage emergence in plots either inoculated or uninoculated with *R. solani* monitored at 2, 3 and 4 weeks after planting.

At 4 weeks after planting, an interesting negative relationship was found between *G. rostochiensis* Pi and emergence in inoculated plots but not in uninoculated plots (Figure 4.6). When regression analysis was undertaken with the data from plots uninoculated with *R. solani*, no relationship was found ($P>0.05$) between *G. rostochiensis* populations and the % emergence of plants. In fact at this particular time, 80% of uninoculated plots had fully emerged regardless of *G. rostochiensis* densities. Contrary to these findings, regression

analysis of inoculated plots revealed a significant relationship between densities of *G. rostochiensis* and % plant emergence ($P<0.01$). While the coefficient of determination (r^2) indicates that this relationship was relatively weak ($r^2 = 0.3455$), a trend clearly existed between the two variates.

Canopy development in inoculated plots was comparably slower than uninoculated plots (Figure 4.7). Infestations of *G. rostochiensis* were also found to be responsible for reducing the rate of canopy development (the average difference between successive daily measurements of percentage canopy ground cover for each plot). A strong and significant negative linear relationship ($P<0.001$, $r^2 =0.7743$) was found between ground cover and *G. rostochiensis* initial population densities in uninoculated plots (Figure 4.8). Although a similar relationship was found in inoculated plots the relationship was less significant and the coefficient of determination far lower ($P<0.01$, $r^2 = 0.3175$).

Table 4.2 Potato cyst nematode Pi in plots from field experiment 2000 (Four-Gates Field, Harper Adams University College) presented in the order that the *R. solani* inoculated and uninoculated plots were paired together

Inoculated plots (eggs g^{-1} soil)	Uninoculated plots (eggs g^{-1} soil)
5.3	9.0
14.8	15.3
27.1	17.4
30.2	34.1
37.5	36.6
44.9	45.2
46.7	49.0
65.3	51.8
67.1	67.1
70.9	72.1
81.6	76.9
85.5	85.8
86.8	86.2
98.4	102.2
125	118.4
133.2	134.9
150.1	147.1
161.0	163.3
209.6	170.9
220.5	175.8

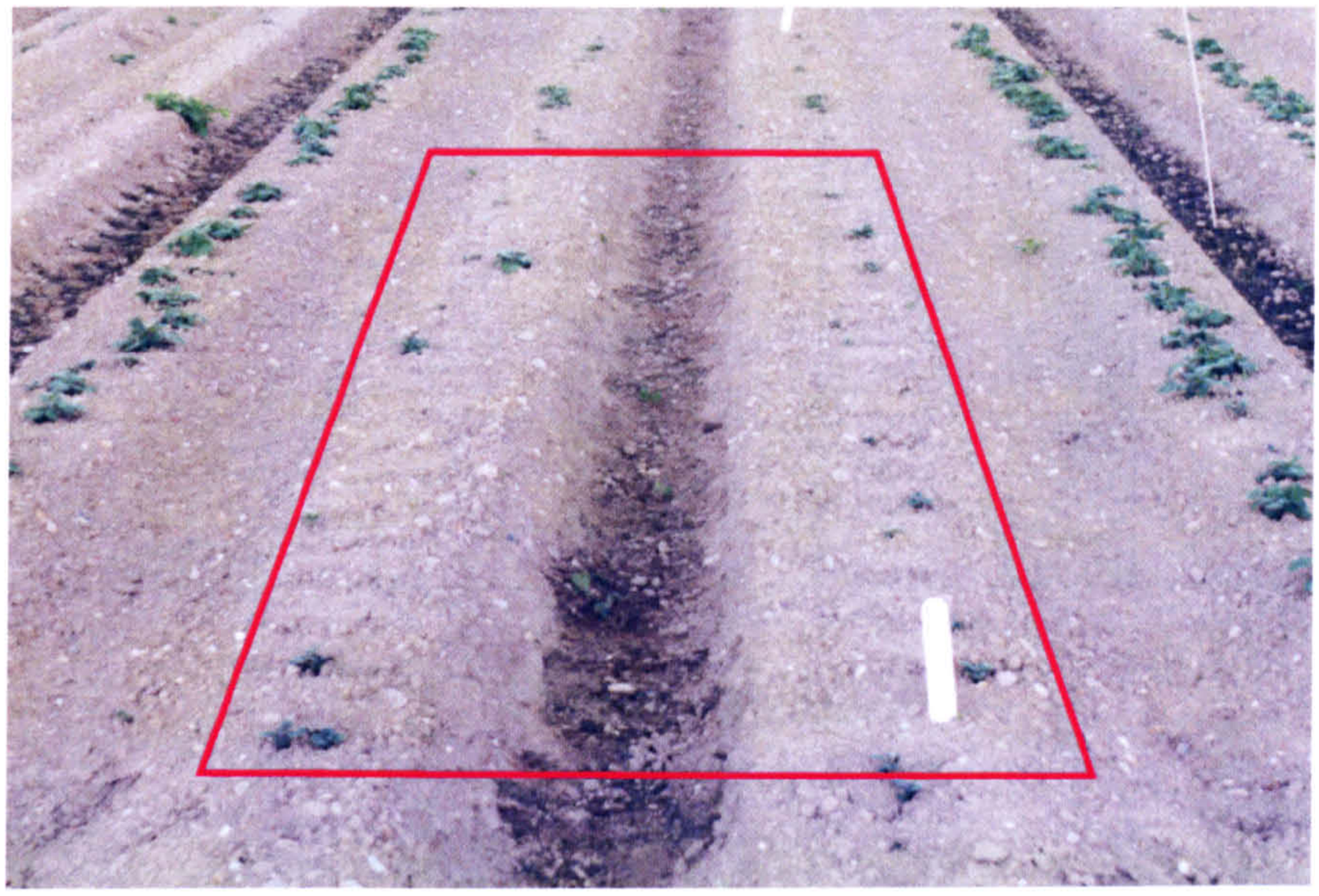


Plate 4.3 An experimental plot from field experiment 2000. The inner two rows highlighted in the plate were inoculated with sand/maize-meal containing *Rhizoctonia solani*

Plate 4.4 An aerial photograph of field experiment 2000 highlighting the delayed emergence in plots inoculated with *Rhizoctonia solani*

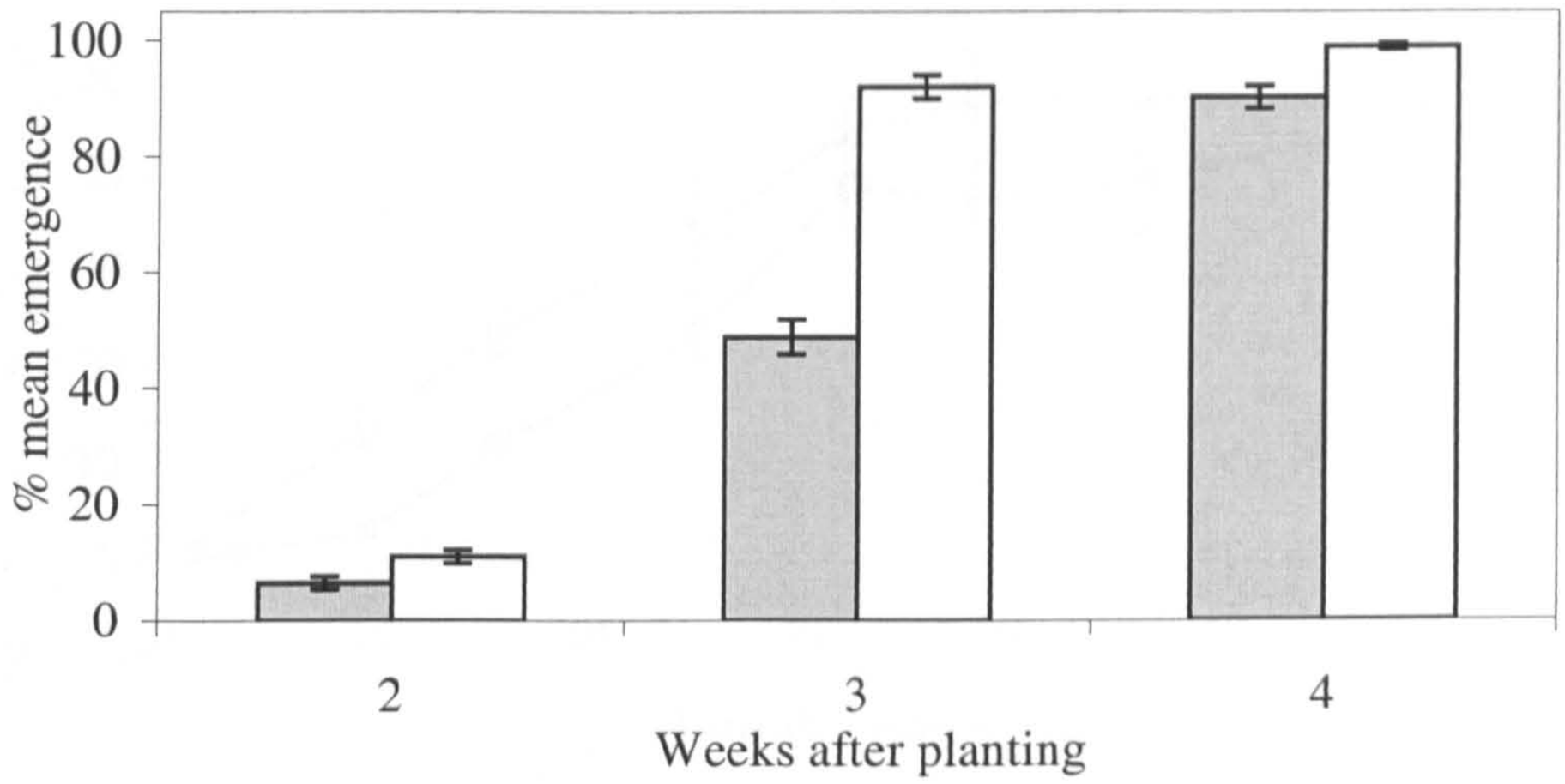


Figure 4.5 % Mean emergence of potato plants (cv. Désirée) in *R. solani* inoculated (■) and uninoculated field plots (□) at weekly intervals after planting field experiment 2000. Error bars show the standard error of the mean

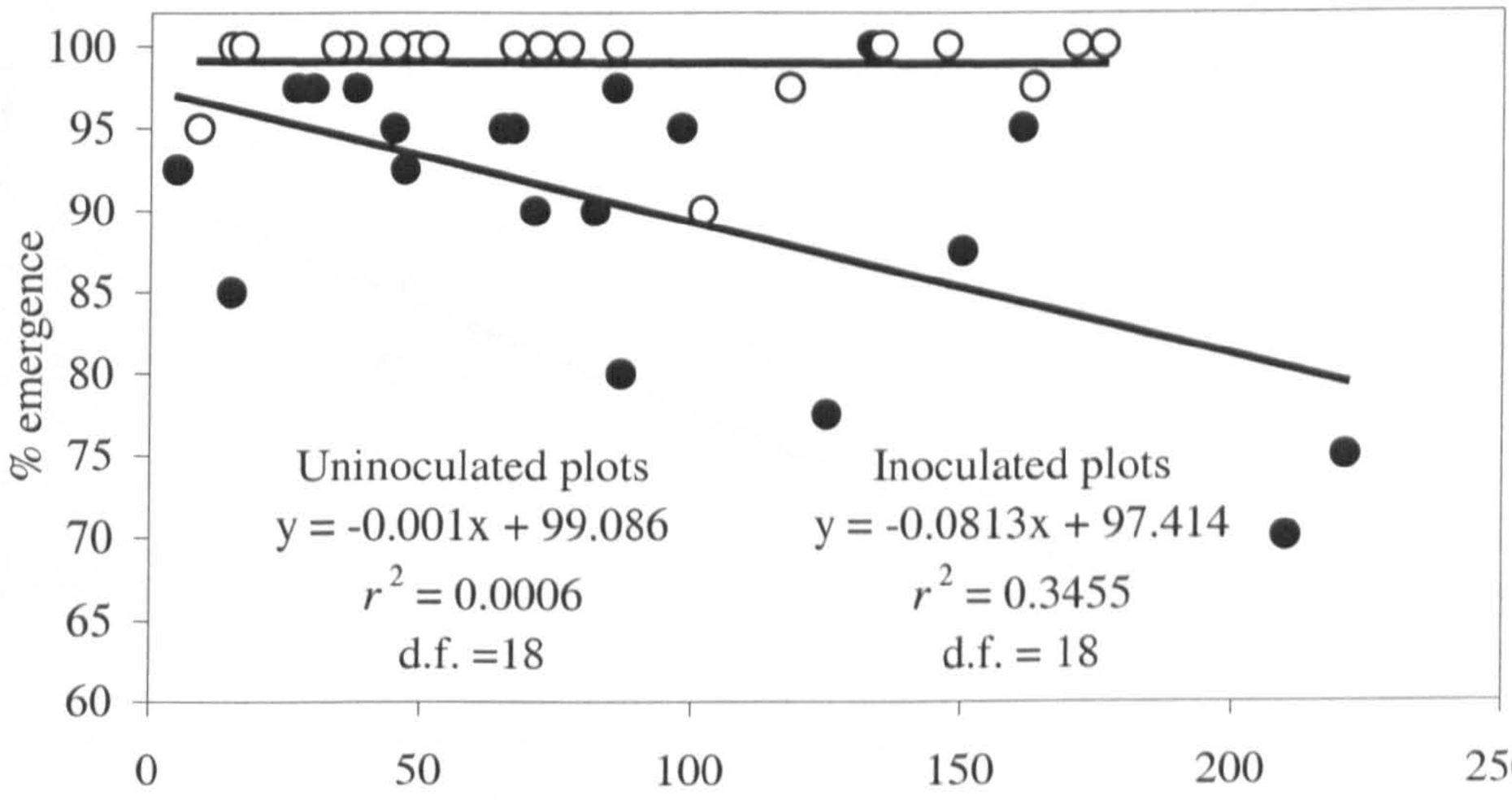


Figure 4.6 The relationship between initial population densities of *G. rostochiensis* and emergence of potato plants (cv. Désirée) in plots inoculated (●) and uninoculated (○) with *R. solani* during 2000

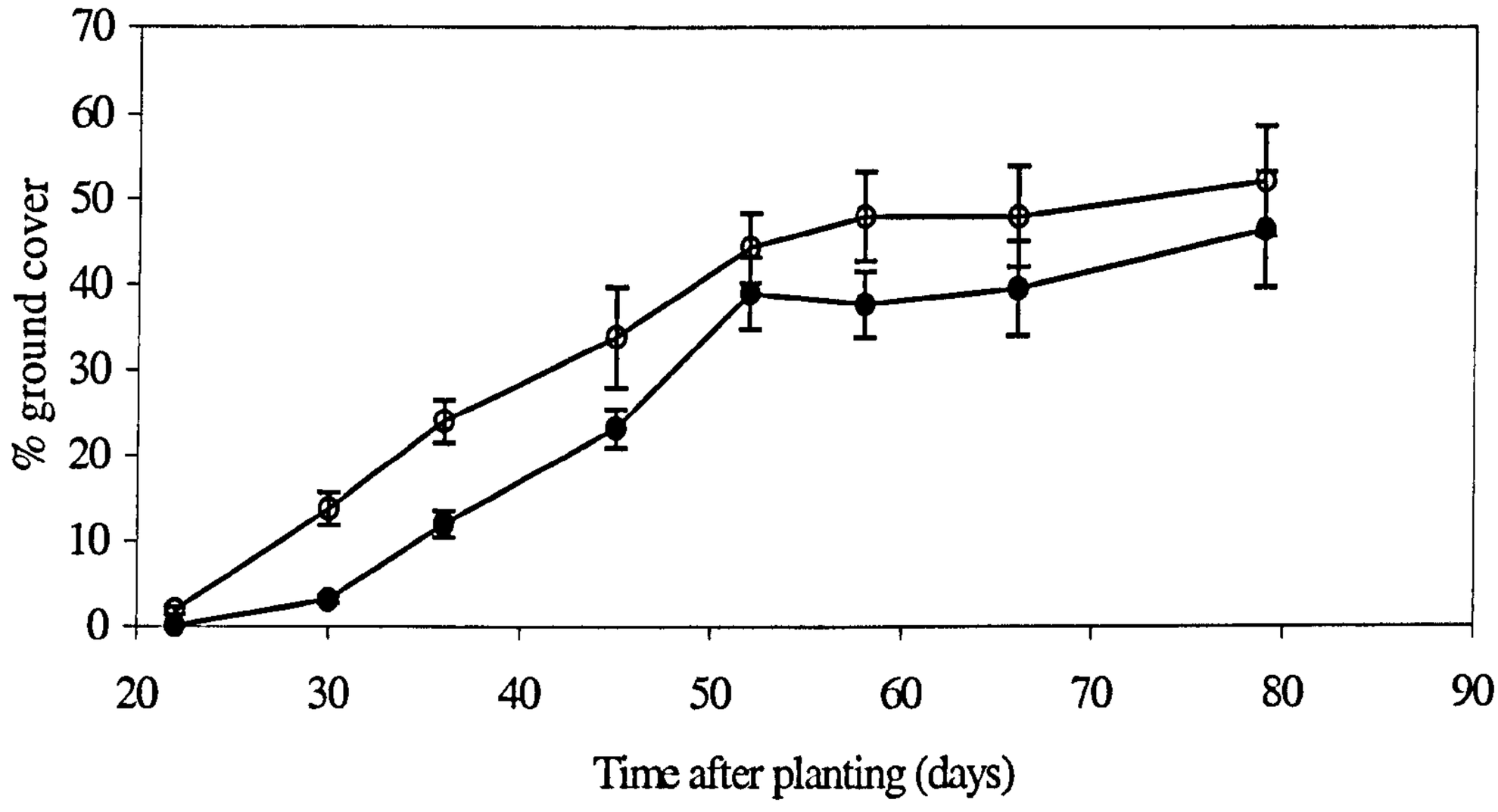


Figure 4.7 Development of the canopy layer in potato (cv. Désirée) plots either inoculated with *R. solani* (●) or left uninoculated (○) 20 to 80 days after planting a field experiment in 2000. Error bars show the standard error of the mean

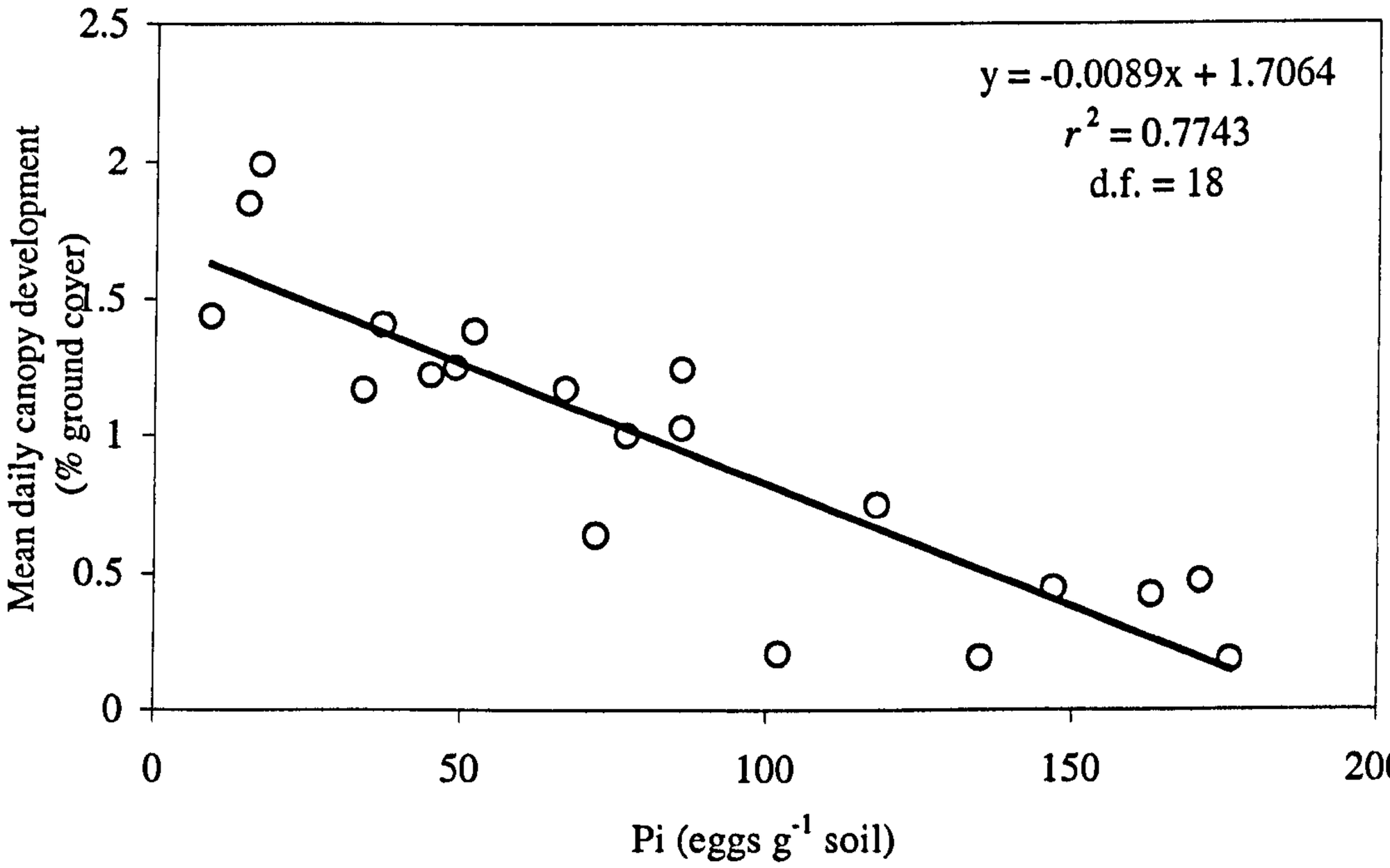


Figure 4.8 The relationship between % mean daily increase in potato canopy (cv. Désirée) and infestation of soil by *G. rostochiensis* in plots left uninoculated during 2000

4.3.1.3 Four-week harvest

The findings presented here are based upon the mean value obtained for each plot. The data were left untransformed unless otherwise stated.

At assessments undertaken on potato plants four weeks after planting, various relationships were found between stem and stolon infection by *R. solani* and the number of *G. rostochiensis* juveniles that had successfully invaded the potato roots. Potato plants collected from plots that were not treated with *R. solani* inoculum were frequently found with symptoms of stem canker, indicating that naturally occurring soil-borne inoculum of *R. solani* was present at the experimental site. Subsequently, *R. solani* disease assessments were undertaken on potato plants from all plots. Plate 4.5 shows the distinct differences in potato plants infected by *R. solani* in plots either inoculated or uninoculated with *R. solani* under high and low infestations of *G. rostochiensis*.

As previously mentioned, the symptoms of stem canker were graded by using an assessment key and calculating the numbers of stems and stolons infected. When *G. rostochiensis* juveniles g^{-1} root was regressed against stem canker index, a significant ($P<0.02$) but weak negative relationship was found for inoculated plots while no relationship was found for uninoculated plots ($P>0.05$) (Figure 4.9). In contrast, there were positive relationships between *G. rostochiensis* Pi densities and stem canker severity in uninoculated plots ($P<0.01$) but no relationship was found in inoculated plots ($P>0.05$) (Figure 4.10).

Plate 4.5 Potato plants (cv. Désirée) harvested 4 weeks after planting field experiment 2000 from field plots with 1. High population densities of *G. rostochiensis* (85.4 eggs g^{-1} soil) and *R. solani* inoculation, 2. Low population densities of *G. rostochiensis* (14.8 eggs g^{-1} soil) and *R. solani* inoculation 3. Low population densities of *G. rostochiensis* left uninoculated (15.3 eggs g^{-1} soil) 4. High population densities of *G. rostochiensis* (85.8 eggs g^{-1} soil) left uninoculated

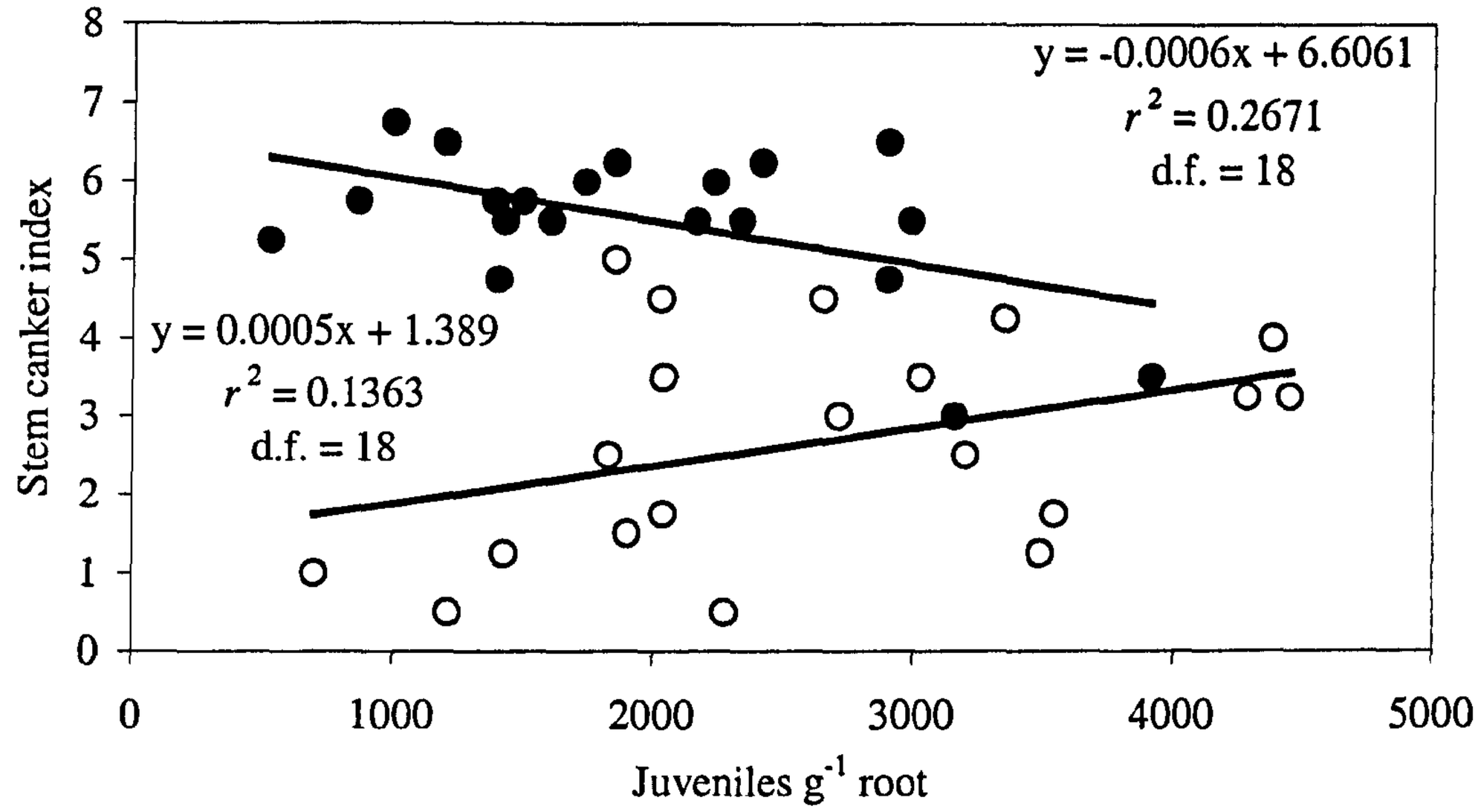


Figure 4.9 The relationship between invasion of potato roots by *G. rostochiensis* juveniles and stem canker severity on potato (cv Désirée) harvested from plots either inoculated with *R. solani* (●) or left uninoculated (○), 4 weeks after planting field experiment 2000

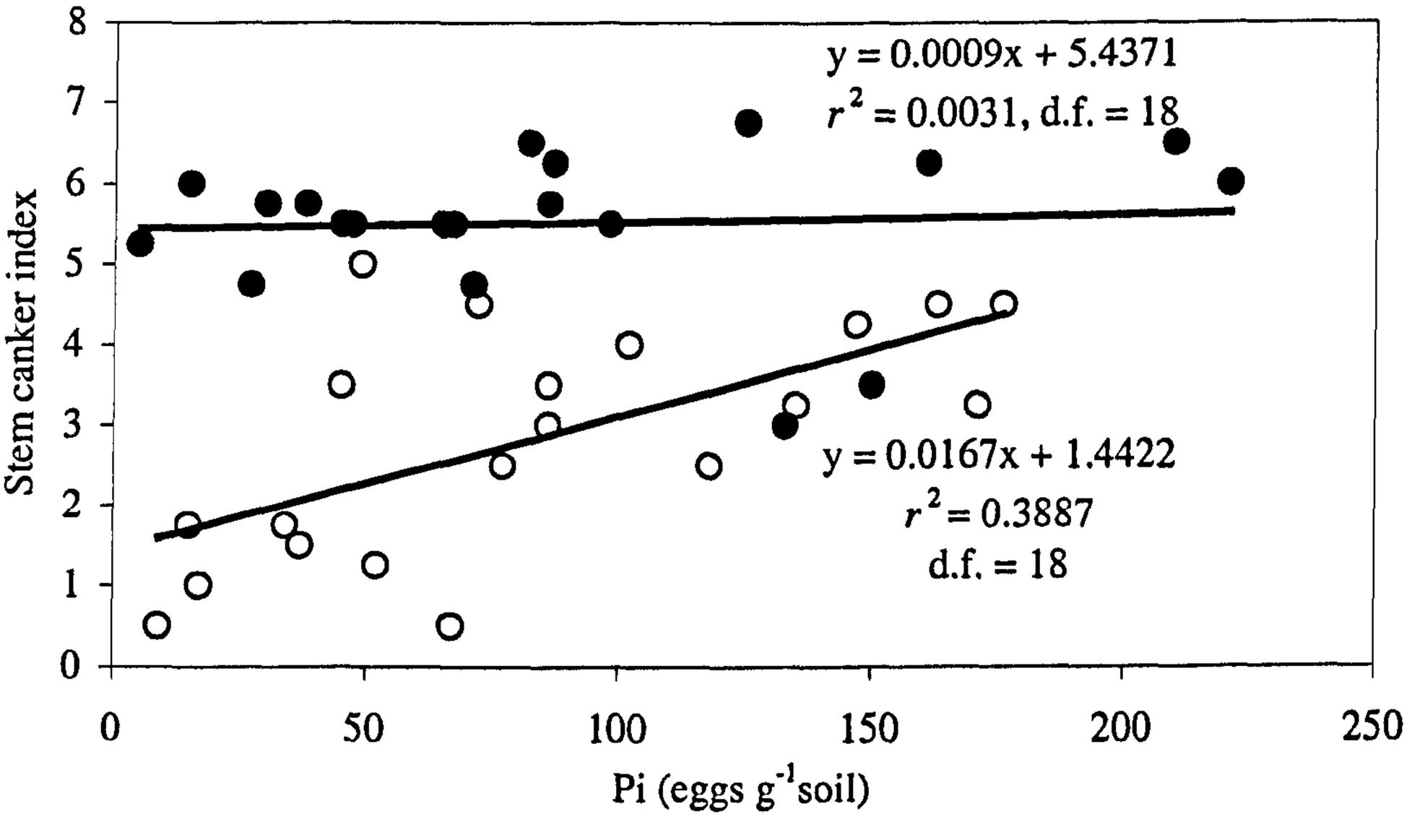


Figure 4.10 The relationship between initial population densities (Pi) of *G. rostochiensis* and stem canker severity on potato (cv Désirée) harvested from plots either inoculated with *R. solani* (●) or left uninoculated (○), 4 weeks after planting field experiment 2000

An alternative way to measure stem canker was to measure the percentage incidence of main stems infected or pruned. Using this approach a significant linear relationship was found between % stems infected with *R. solani* and *G. rostochiensis* Pi densities in uninoculated plots ($P<0.004$). Furthermore by fitting a critical exponential line to the data, a moderate relationship was found (Figure 4.11). No relationships were found for inoculated plots.

The incidence of stolons infected by *R. solani* (stolon canker) was significantly correlated to both the number of juvenile *G. rostochiensis* invading the potato roots (Figure 4.12) and Pi in both inoculated ($P<0.05$) and uninoculated ($P<0.05$) plots (Figure 4.13). In the relationship between Pi and stolons infected, there was a significant difference between the regression lines ($P<0.05$) for inoculated and uninoculated plots.

Multiple regression analysis was conducted to determine the individual and combined effects of *R. solani* diseases and *G. rostochiensis* infestation upon the morphological features of potato plants. The majority of these analyses was found to be non-significant and are therefore not presented.

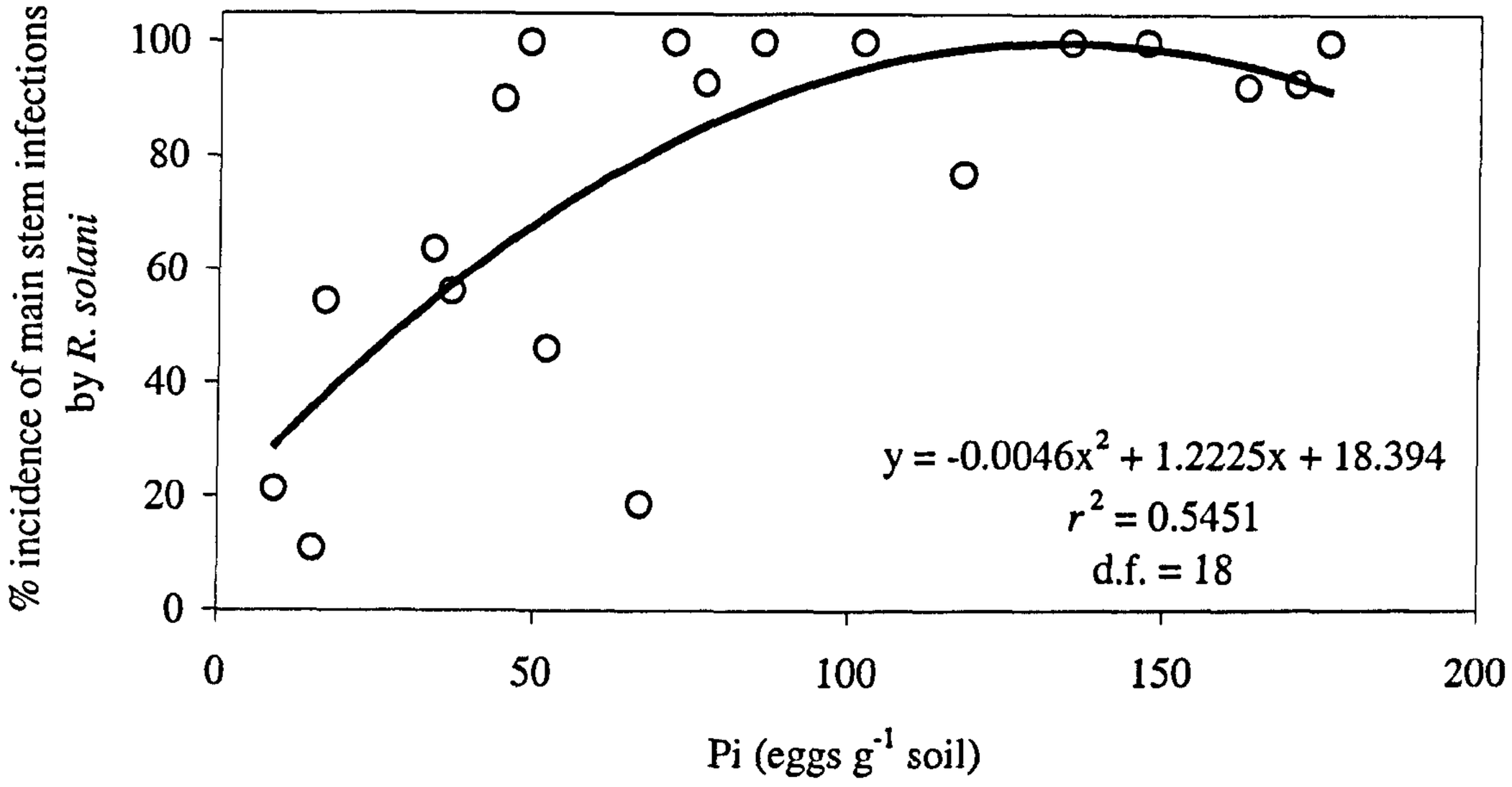


Figure 4.11 The relationship between initial population densities (Pi) of *G. rostochiensis* and the incidence of potato (cv. Désirée) main stems infected by *R. solani* in plots left uninoculated, 4 weeks after planting field experiment 2000

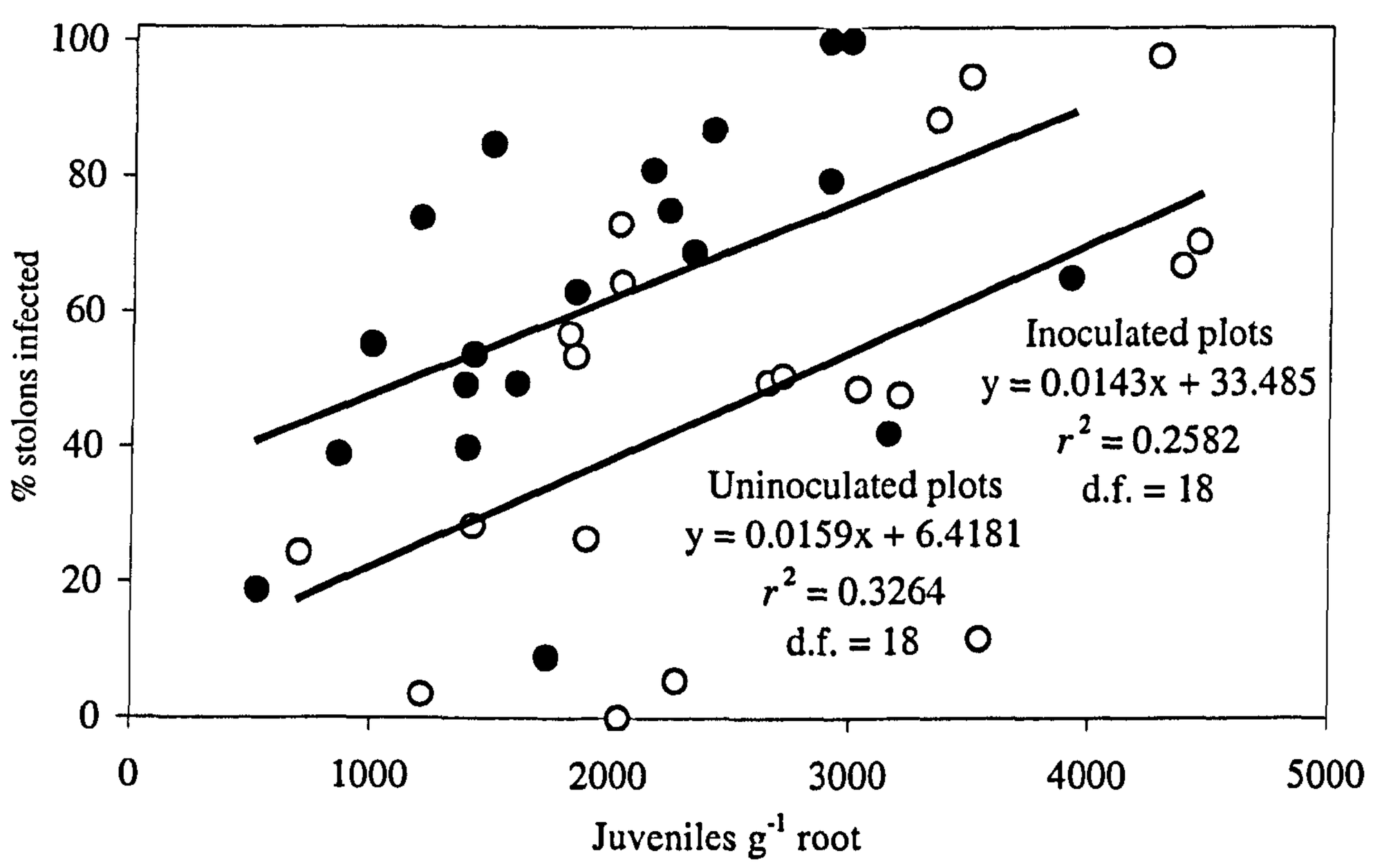


Figure 4.12 The relationship between invasion of potato (cv. Désirée) roots by *G. rostochiensis* juveniles and the infection of stolons by *R. solani* in plots either inoculated with *R. solani* (●) or left uninoculated (○), four weeks after planting field experiment 2000

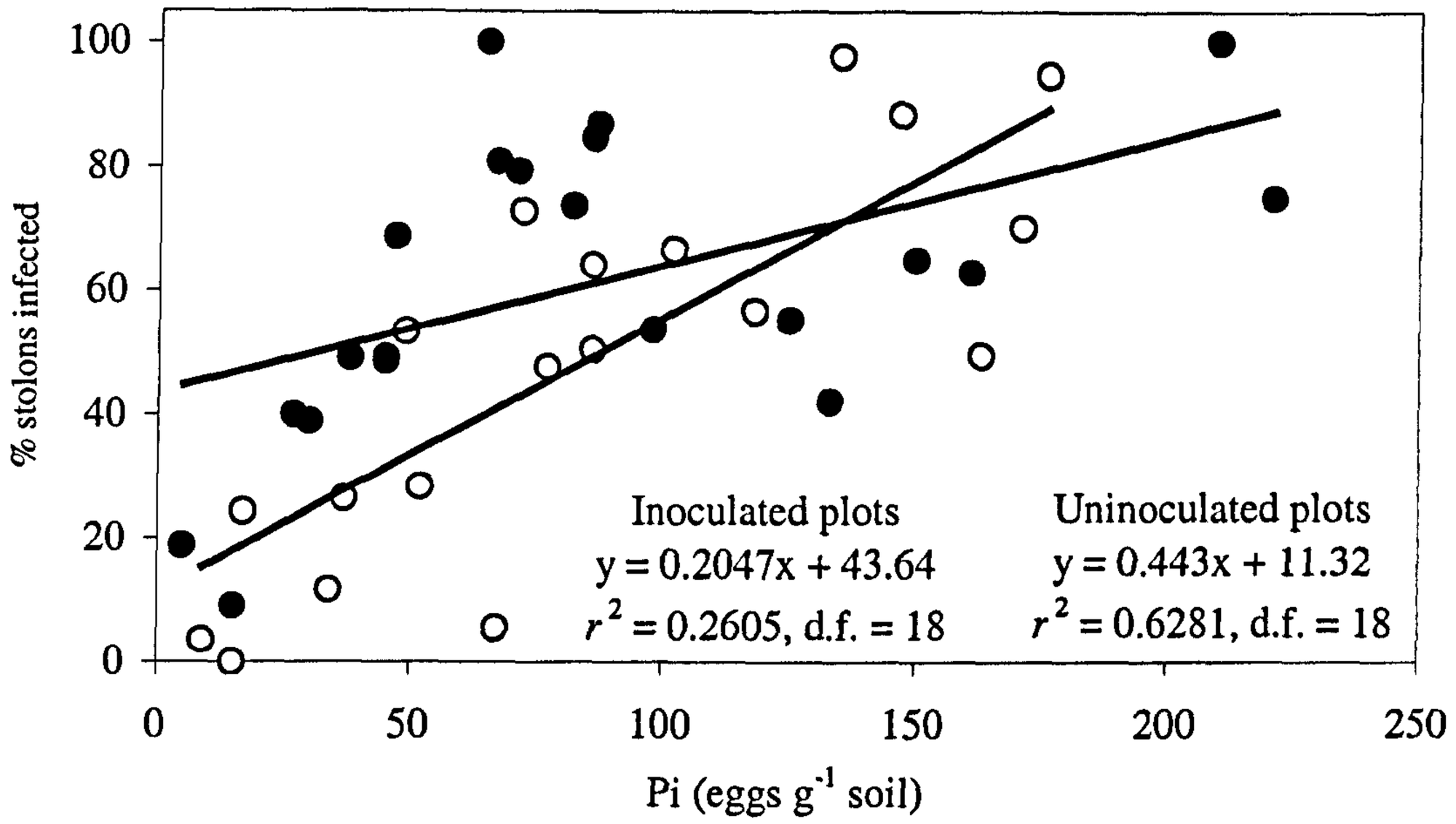


Figure 4.13 The relationship between *G. rostochiensis* initial population densities (Pi) and the incidence of stolons infected by *R. solani* for potato plants (cv. Désirée) harvested from plots either inoculated with *R. solani* (●) or left uninoculated (○), four weeks after planting field experiment 2000

4.3.1.4 Six-week harvest

Linear regression analysis revealed a significant relationship between stolon infections and invasion of potato roots by *G. rostochiensis* juveniles in inoculated plots. However, by examining the scatter diagram of these two variates, it was apparent that the relationship was non-linear. Using the curve-fitting tool in Gentstat © the relationship appeared to be best defined by a double-exponential fit, which produced a highly significant relationship ($P<0.003$) between the two variates (Figure 4.14).

No relationship was found between stolons infected and *G. rostochiensis* juveniles in uninoculated plots. However, as with the 4-week harvest, a significant relationship was found between main stems infected and *G. rostochiensis* initial populations ($P<0.001$, $r^2 = 0.58$) or juvenile invasion ($P<0.003$, $r^2 = 0.56$) in uninoculated plots when a critical exponential curve was fitted. No further relationships were found between *G. rostochiensis* and *R. solani* diseases at this harvest.

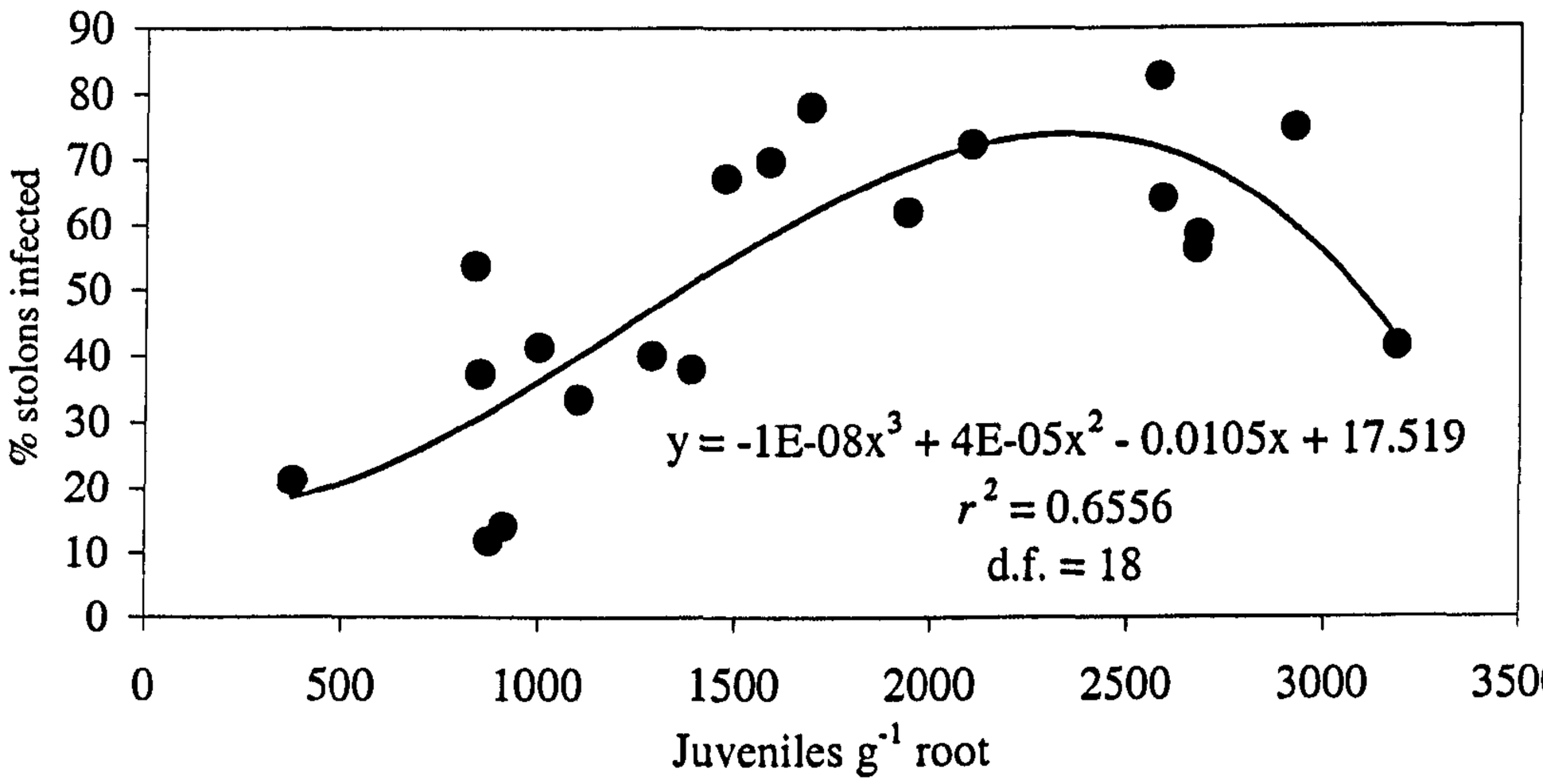


Figure 4.14 The relationship between invasion of potato (cv. Désirée) roots by *G. rostochiensis* juveniles and infection of stolons by *R. solani* in plots inoculated with *R. solani*, 6 weeks after planting field experiment 2000

4.3.1.5 Eight-week harvest

A weak relationship was found between stolon infections and the invasion of roots by *G. rostochiensis* ($P<0.05$, $r^2 = 0.24$) in inoculated plots and uninoculated plots ($P<0.01$, $r^2 = 0.34$). No further relationships were found between *R. solani* diseases and *G. rostochiensis* infestations.

So far, the analyses undertaken have focussed upon relationships between nematode infestations and the development of *R. solani* diseases. However, consideration also needs to be given as to whether nematode development is disrupted, facilitated or unaffected by *R. solani* infections. Linear regression analysis of the first two harvests did not give any indication that the severity of stem or stolon canker had any bearing on nematode development. Such analyses included investigating the proportion (%) of juvenile *G. rostochiensis* developing into females and the proportion of juveniles (%) that were in the final two juvenile moults (i.e. Juvenile stages 4 and 5). At 8 weeks after planting, a weak negative relationship ($P<0.05$) was found between the percentage of female *G. rostochiensis* within juvenile stages 4 and 5 (J4 and J5) and % stolons infected by *R. solani* in inoculated plots only (Figure 4.15). Interestingly, a similar negative relationship was also obtained when total juveniles per gram of root (*G. rostochiensis* root invasion) was regressed against % female J4/J5 ($P<0.005$) (Figure 4.16). In contradiction to the simple linear regression analyses undertaken, multiple regression analysis revealed that neither *G. rostochiensis* root invasion nor % stolons infected had any significant effect on the proportion of *G. rostochiensis* juveniles developing into females.

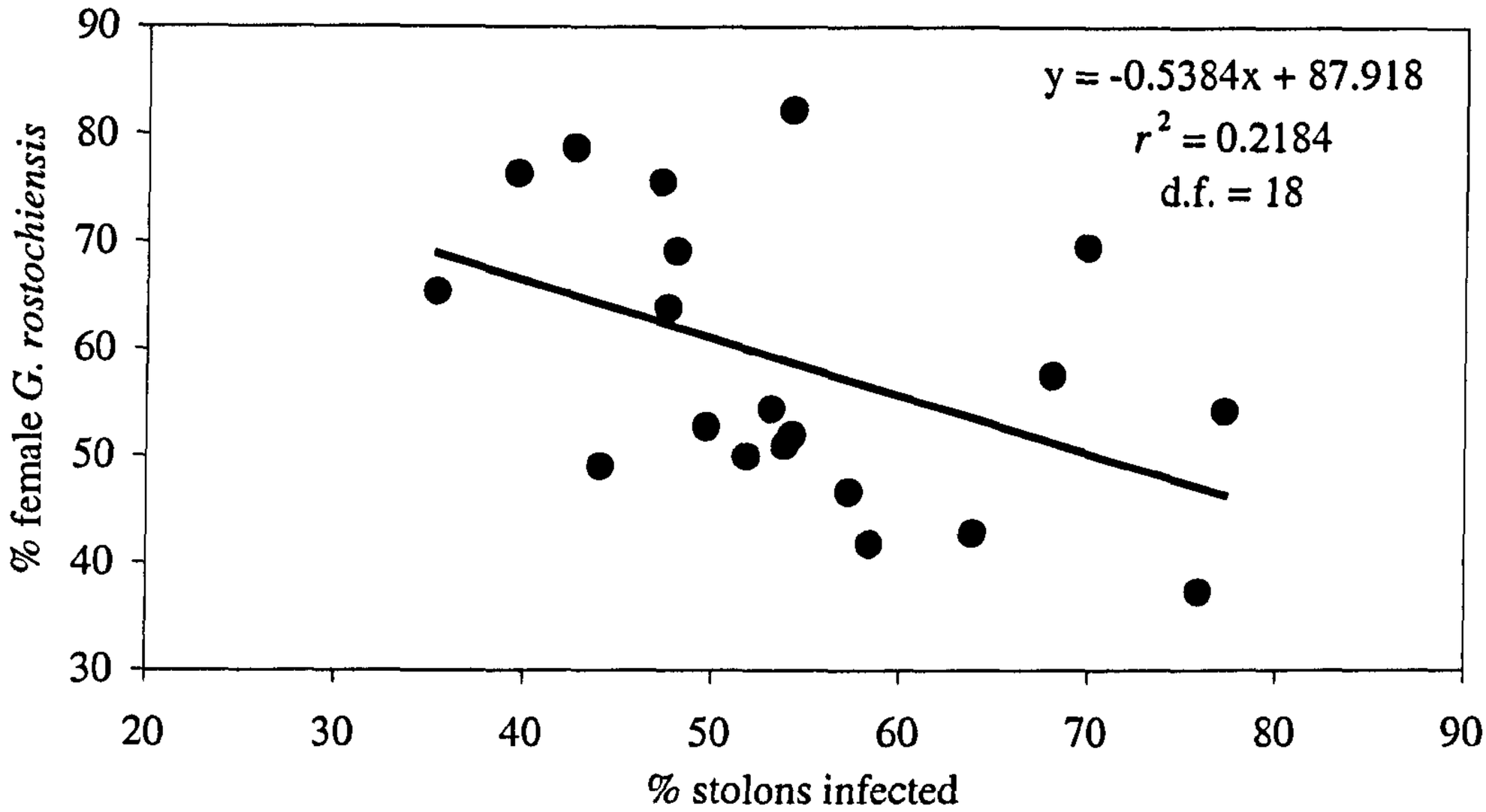


Figure 4.15 The relationship between potato (cv. Désirée) stolons infected by *R. solani* and the proportion of *G. rostochiensis* juveniles (J4 and J5) developing into females in plots inoculated with *R. solani*, 8 weeks after planting field experiment 2000

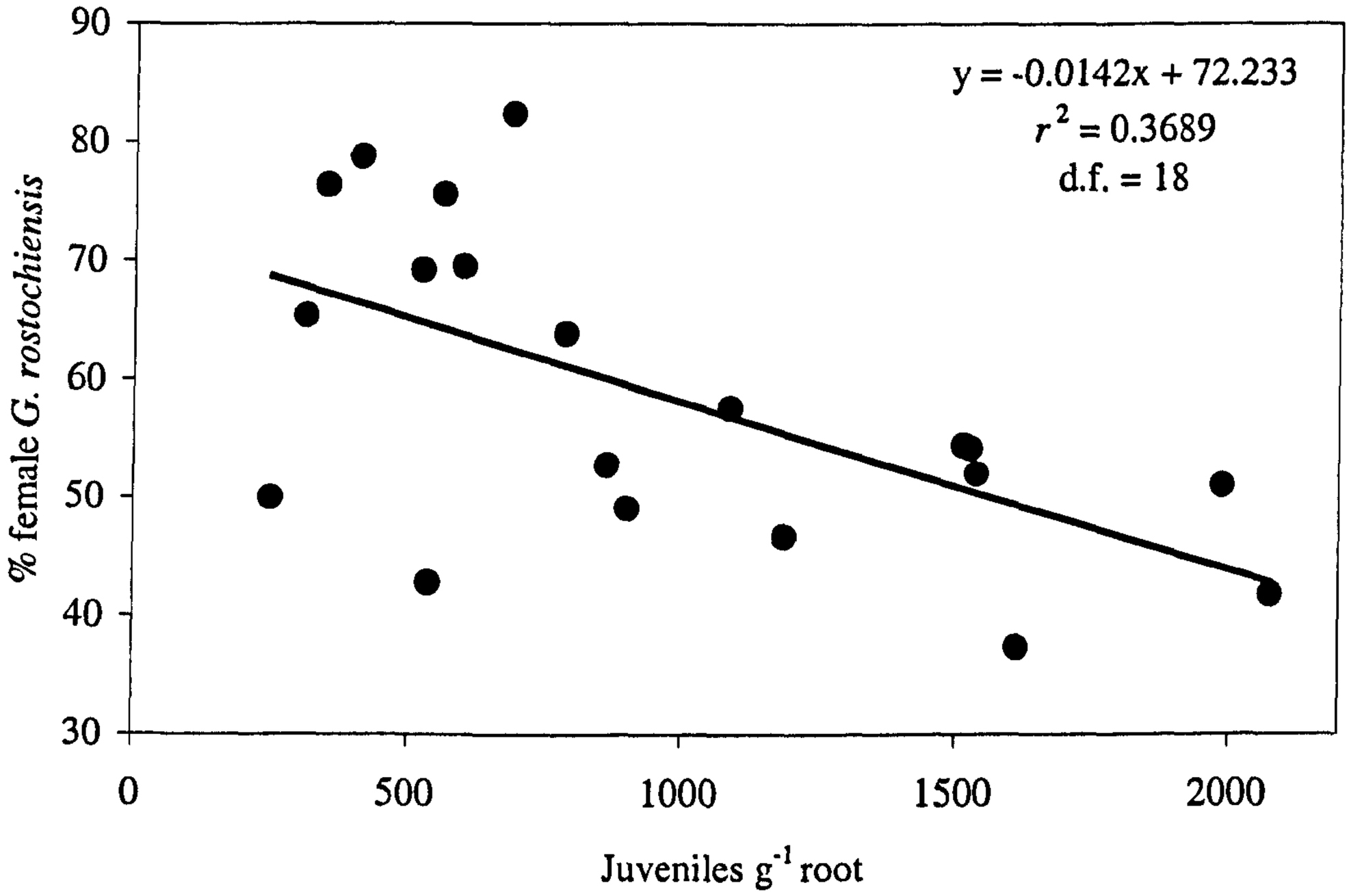


Figure 4.16 The relationship between the total densities of *G. rostochiensis* juveniles (J4 and J5) found within the roots of potato plants (cv. Désirée) and the proportion developing into females in plots inoculated with *R. solani*, 8 weeks after planting field experiment 2000

4.3.1.6 *G. rostochiensis* moults at 4, 6 and 8-week harvests

The numbers and proportions (%) of *G. rostochiensis* juveniles stages (moults), at each sampling date for plots either inoculated with *R. solani* or left uninoculated are presented in Table 4.3. At 4 weeks after planting, a high proportion of juvenile stages J2-J3 were observed in plots either inoculated with *R. solani* or left uninoculated, whereas at 6 and 8 weeks after planting, there was a shift towards the later moults (J4-J5).

Table 4.3 The distribution of *G. rostochiensis* juvenile stages (moults) recovered from the roots of potato plants (cv. Désirée) at 4, 6 and 8 weeks after planting in field plots either inoculated with *R. solani* (a) or left uninoculated (b) in field experiment 2000

(a)

	Mean number and % (in parentheses) of *G. rostochiensis* juveniles recovered		
G. rostochiensis moults	4 weeks	6 weeks	8 weeks
J2	771.6 (39.1)	392.2 (22.1)	73.3 (7.6)
J3	804.1 (40.7)	590.0 (33.2)	307.4 (31.8)
Sum J4+J5 ♂	198.2 (10.0)	433.8 (24.4)	287.4 (29.8)
Sum J4+J5 ♀	172.5 (8.7)	380.1 (20.27)	346.6 (35.9)

(b)

	Mean number and % (in parentheses) of *G. rostochiensis* juveniles recovered		
G. rostochiensis moults	4 weeks	6 weeks	8 weeks
J2	1172 (43.4)	301.5 (13.9)	78.5 (9.9)
J3	882.4 (32.7)	524.1 (27.6)	211.6 (26.7)
Sum J4+J5 ♂	354.1 (13.2)	641.3 (33.8)	181.3 (22.9)
Sum J4+J5 ♀	290.2 (10.8)	430.4 (22.7)	321.3 (40.5)

4.3.1.7 Final harvest (yield)

In order to estimate the factors affecting yield, tuber size (grade) and black scurf development, means of previously recorded measurements of stolon canker and *G. rostochiensis* juvenile invasion (data collected in 4, 6, and 8 week harvests) were used in regression analyses. Table 4.4 and 4.5 provide summaries of the relationships found in inoculated and uninoculated plots

Table 4.4 A summary of simple linear regression analyses comparing measurements taken during the final harvest with mean infection of potato (cv. Désirée) stolons by *R. solani* or mean *G. rostochiensis* juvenile invasion of potato roots in plots inoculated with *R. solani* during field experiment 2000

Explanatory Variate	Response variate	Significance (*P*)	Standard error (S.E.)	Coefficient of determination (r^2)
% Stolons infected	Tuber yield	<0.001	0.0146	0.6665
	Tuber number	0.443	0.243	0.033
	% Tubers <45 mm	0.265	0.229	0.0684
	% Tubers 45-65 mm	0.293	0.219	0.0613
	% Tubers 65-85 mm	0.447	0.0335	0.0325
	Black scurf	0.312	0.0061	0.0568
Juveniles g^{-1} root	Tuber yield	<0.001	0.0004	0.4707
	Tuber number	0.152	0.0056	0.1106
	% Tubers <45 mm	0.610	0.0056	0.0147
	% Tubers 45-65 mm	0.610	0.0053	0.0145
	% Tubers 65-85 mm	0.871	0.0008	0.0015
	Black scurf	0.129	0.0002	0.074

d.f. = 18

Table 4.5 A summary of simple linear regression analyses comparing measurements taken during the final harvest with mean infection of potato (cv. Désirée) stolons by *R. solani* or mean *G. rostochiensis* juvenile invasion of potato roots in plots left uninoculated during field experiment 2000

Explanatory Variate	Response variate	Significance (*P*)	Standard error (S.E.)	Coefficient of determination (r^2)
% Stolons infected	Tuber yield	<0.001	0.291	0.5172
	Tuber number	0.052	0.0166	0.1936
	% Tubers <45 mm	0.654	0.240	0.0114
	% Tubers 45-65 mm	0.660	0.207	0.011
	% Tubers 65-85 mm	0.672	0.0385	0.0102
	Black scurf	0.012	0.004	0.2996
Juveniles g^{-1} root	Tuber yield	<0.001	0.0004	0.6157
	Tuber number	0.007	0.0065	0.3388
	% Tubers <45 mm	0.651	0.0059	0.0116
	% Tubers 45-65 mm	0.591	0.0051	0.0163
	% Tubers 65-85 mm	0.942	0.0009	0.0003
	Black scurf	0.029	0.0001	0.2376

d.f. =18

Since simple linear regression indicated relationships between yield and both mean juvenile invasion and mean stolon infections, stepwise multiple regression models were employed to further investigate the influence of these two variates. Multiple regression analysis revealed that stolon infections had a significant relationship with yield in inoculated plots ($P<0.005$) whereas *G. rostochiensis* infestation (juvenile invasion) was not significant ($P>0.05$) in this model. In uninoculated plots, the converse was found where juvenile invasion had a

significant negative relationship with yield ($P<0.01$) whereas stolon infection was significant but to a weaker degree ($P<0.05$).

The % area coverage of black scurf on tubers (square root transformed) was not found to be related to invasion of *G. rostochiensis* juveniles in inoculated plots, however, a weak positive relationship was found in uninoculated plots ($P<0.05$, $r^2 = 0.2376$). Black scurf was also found to be weakly related to stolon canker in uninoculated plots ($P<0.05$, $r^2 = 0.2996$).

At the final yield harvest, the possible effect of *R. solani* diseases on *G. rostochiensis* population densities was considered using *G. rostochiensis* multiplication rates (equation 4).

$$\frac{\text{Final population densities (Pf)}}{\text{Initial population densities (Pi)}} = \text{Pf/Pi (Multiplication rate)} \qquad \text{equation (4)}$$

Mean stolon infection (from 4, 6 and 8 week harvests) showed a moderate negative correlation with square root transformed Pf/Pi in inoculated ($r = -0.596$) and uninoculated ($r = -0.695$) plots. Subsequent regression analysis revealed significant relationships between mean stolon infection and Pf/Pi in inoculated ($P<0.006$) and uninoculated ($P<0.001$) plots (Figures 4.17 and 4.18). Similar regression analyses with mean juvenile invasion (from 4, 6 and 8 week harvests) and square root transformed Pf/Pi were also found to produce negative linear relationships for inoculated ($P<0.01$, $r^2 = 0.3329$) and uninoculated ($P<0.001$, $r^2 = 0.6358$) plots (Figures 4.19 and 4.20). Multiple regression analyses were undertaken to further investigate the relationships between the explanatory variates and the multiplication rate (Table 4.6).

Table 4.6 Multiple regression analysis results showing the response of *G. rostochiensis* multiplication to mean *G. rostochiensis* juvenile densities and the infection of stolons by *R. solani* on potato plants (cv. Désirée) harvested 4, 6 and 8 weeks after planting field experiment 2000

Treatment	Explanatory variates	Significance (*P*)	Standard error (S.E.)
Inoculated plots (d.f. = 18)	Juveniles g^{-1} root	0.045	0.0014
	% Stolons infected	0.031	0.0336
	Juveniles g^{-1} root * % Stolons infected	0.068	0.00002
Uninoculated plots (d.f. = 18)	Juveniles g^{-1} root	0.017	0.0005
	% Stolons infected	0.067	0.0245
	Juveniles g^{-1} root * % Stolons infected	0.191	0.00001

As seen from Table 4.6, both *G. rostochiensis* juvenile densities within the roots and % stolon infections caused by *R. solani* were found to be related to the multiplication rate of *G. rostochiensis* in inoculated plots. In comparison, relationships between *G. rostochiensis* juvenile densities and the multiplication rate were only found in uninoculated plots. No relationships were found between the interacting explanatory variates *G. rostochiensis* juvenile densities within the roots, % stolon infections caused by *R. solani* and the multiplication rate of *G. rostochiensis*.

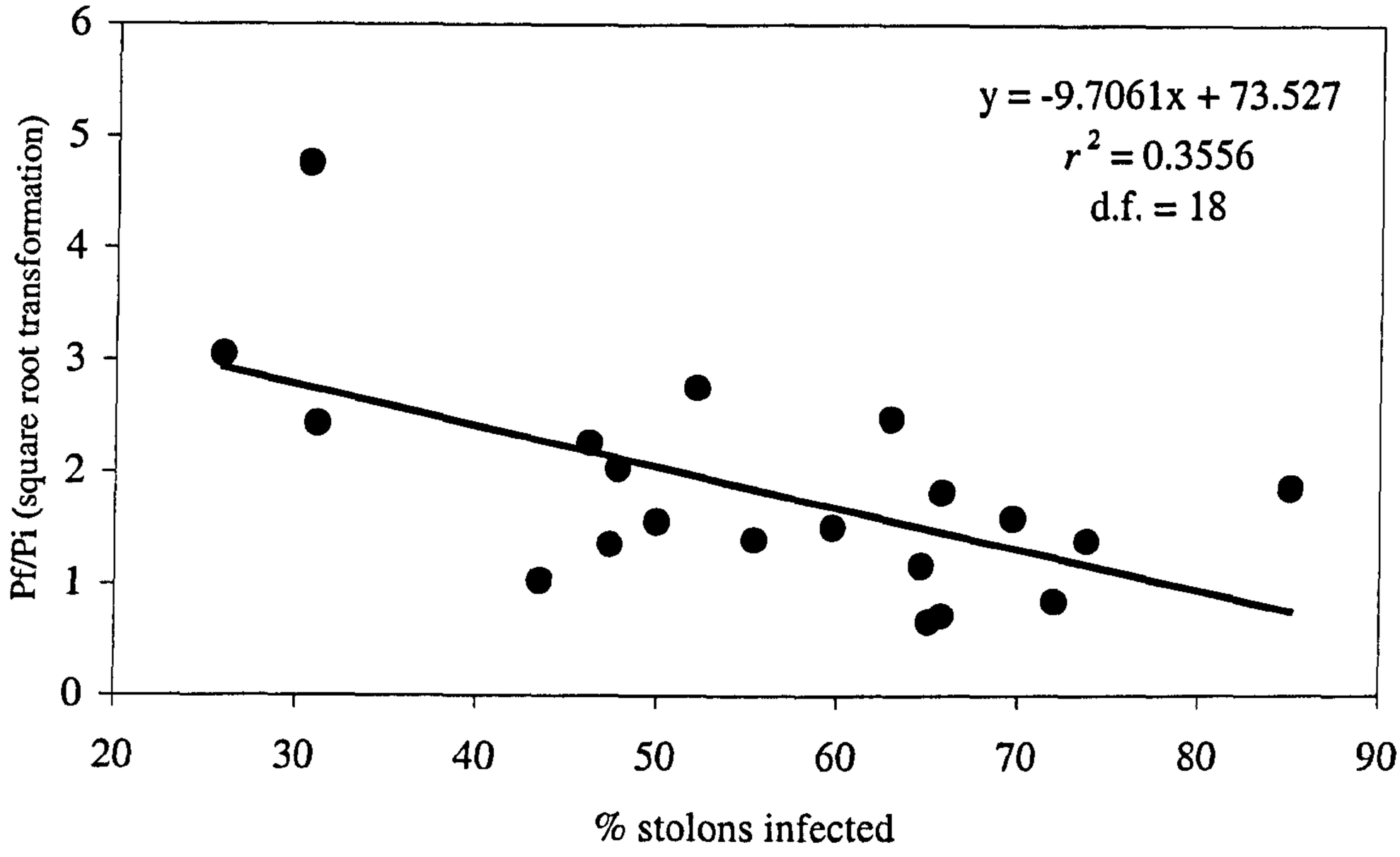


Figure 4.17 The relationship between mean incidence of potato (cv. Désirée) stolons infected by *R. solani* (4, 6 and 8 week harvests) and the multiplication rate of *G. rostochiensis* (Pf/Pi) in plots inoculated with *R. solani*, from field experiment 2000

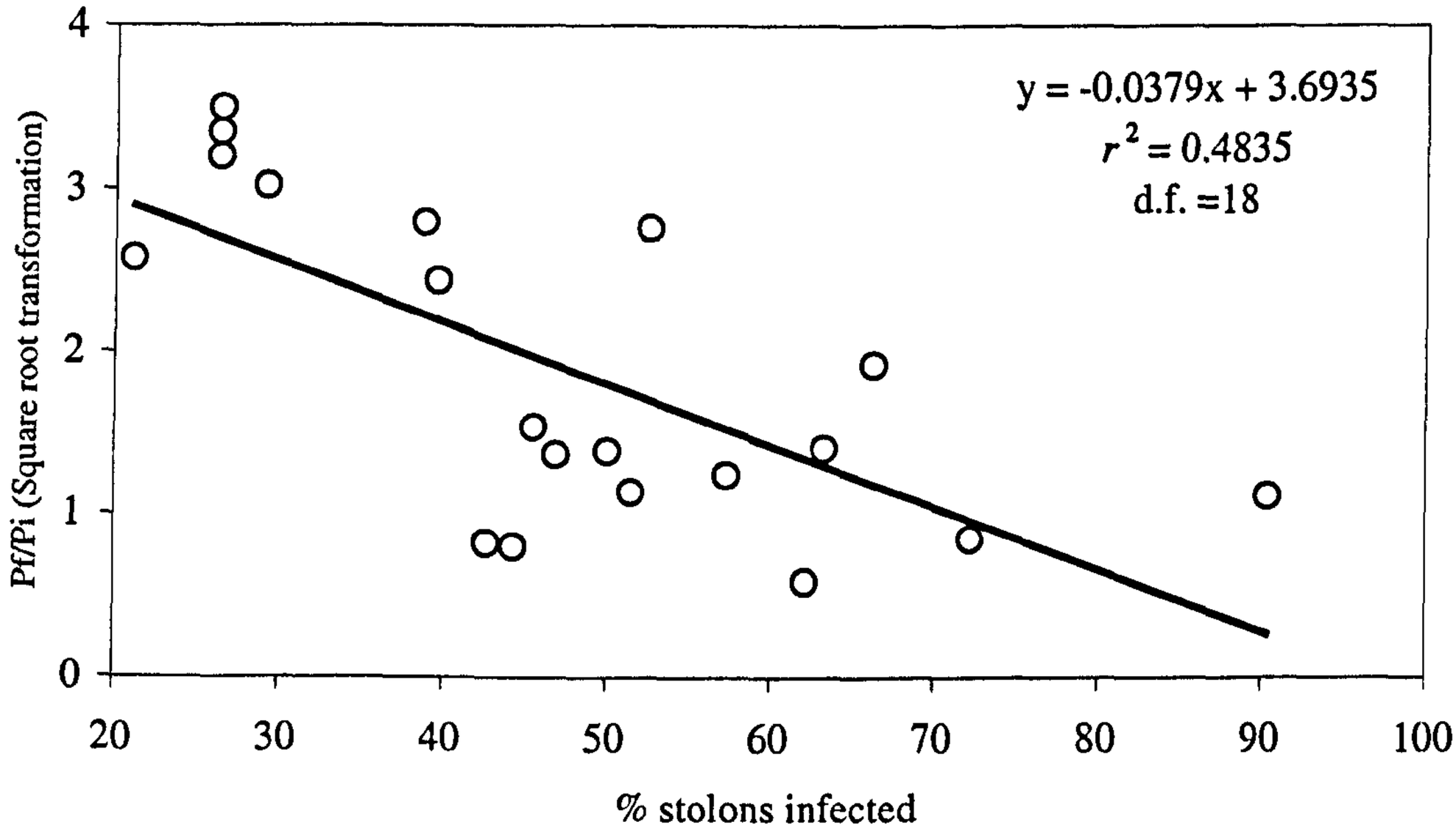


Figure 4.18 The relationship between mean incidence of potato (cv. Désirée) stolons infected by *R. solani* (4, 6 and 8 week harvests) and the multiplication rate of *G. rostochiensis* (Pf/Pi) in plots left uninoculated, from field experiment 2000

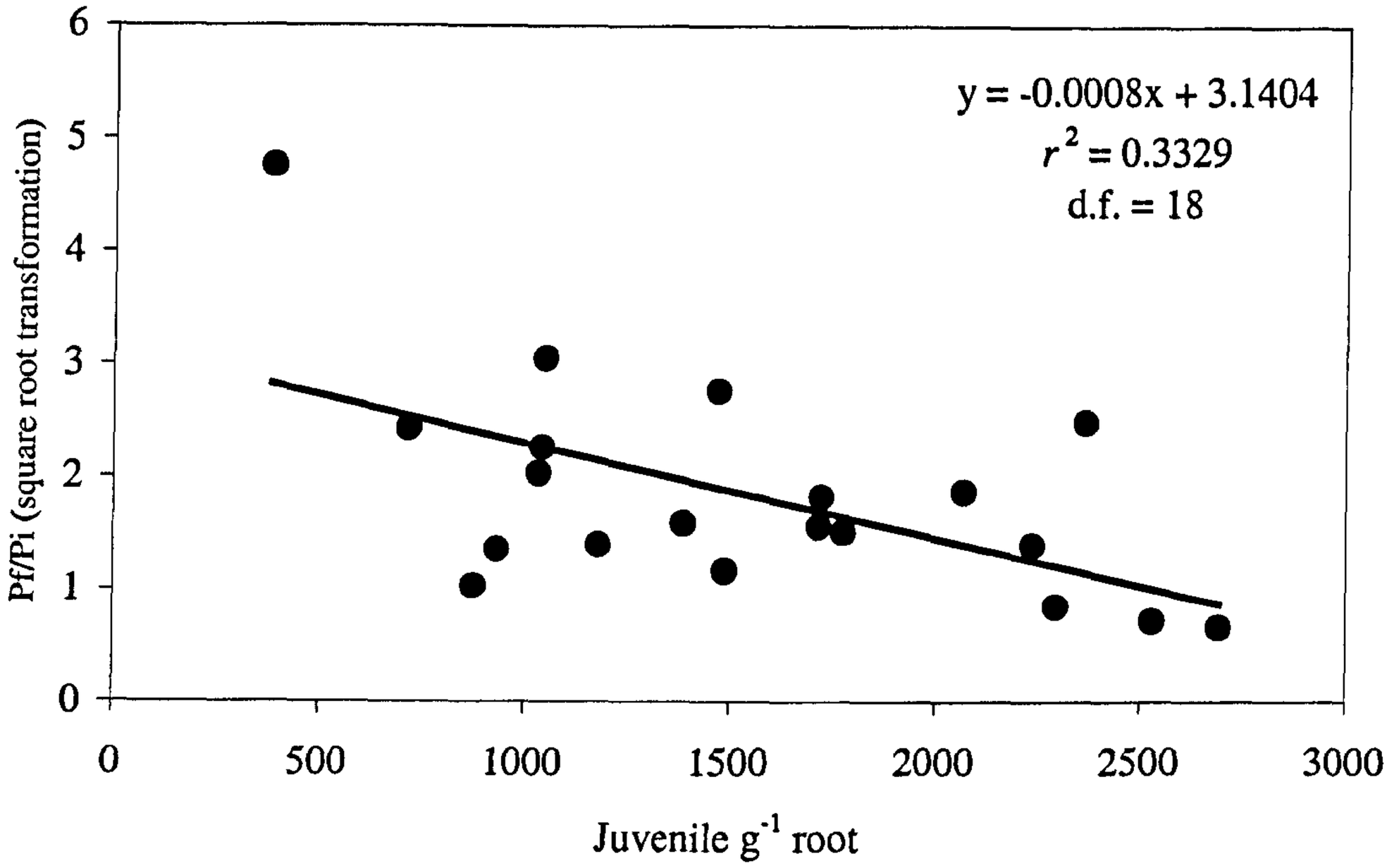


Figure 4.19 The relationship between mean juvenile invasion of potato roots (cv. Désirée) by *G. rostochiensis* (4, 6 and 8 week harvests) and the multiplication rate of *G. rostochiensis* (Pf/Pi) in plots inoculated with *R. solani*, from field experiment 2000

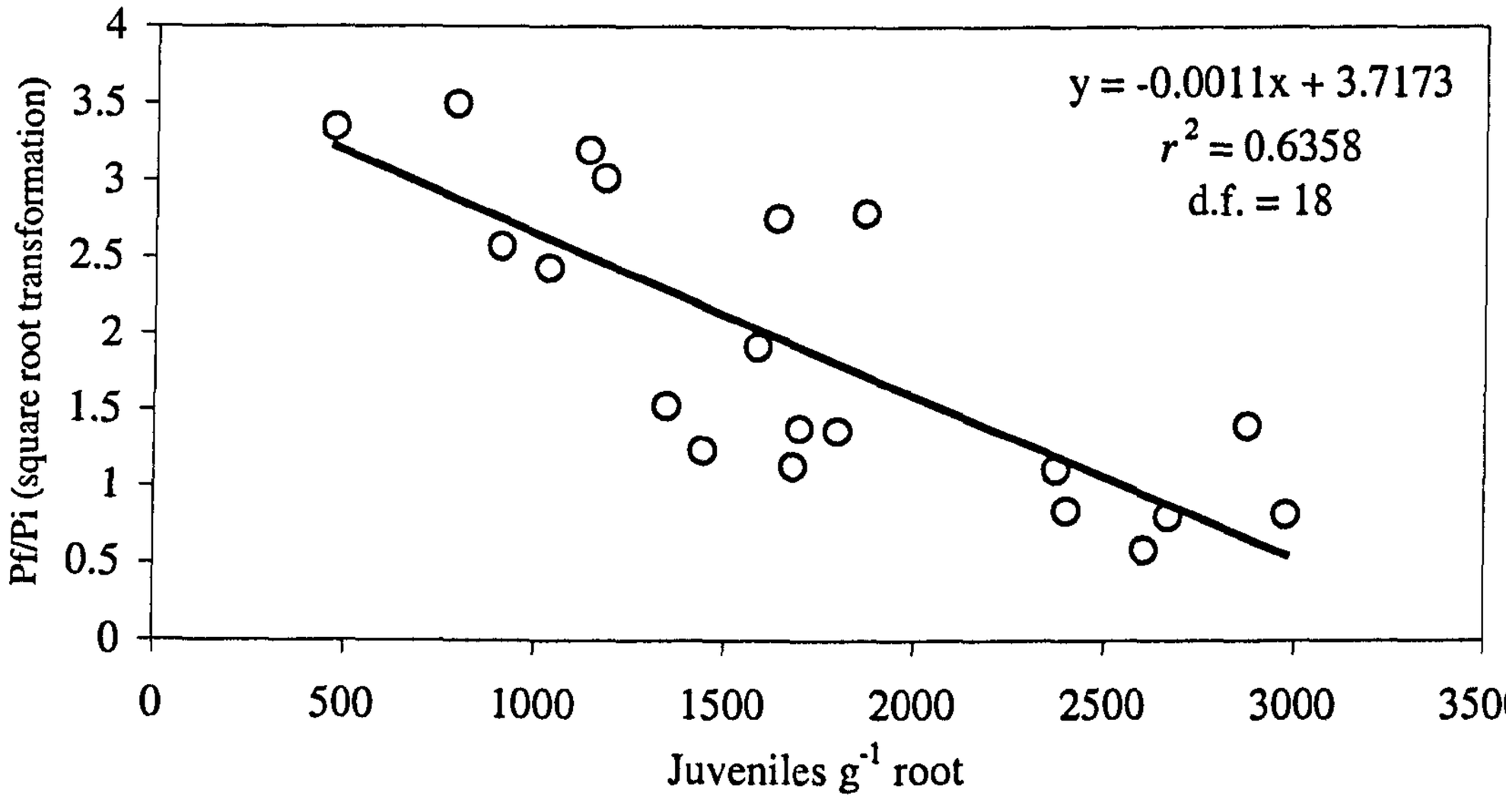


Figure 4.20 The relationship between mean juvenile invasion of potato roots (cv. Désirée) by *G. rostochiensis* (4, 6 and 8 week harvests) and the multiplication rate of *G. rostochiensis* (Pf/Pi) in plots left uninoculated, from field experiment 2000

4.3.2 Field experiment 2001

Potato cyst nematode initial densities are shown in Table 4.7 Polymerase chain reaction assays revealed that the majority of plots were made up of pure populations of *G. rostochiensis* with the exception of two plots where mixed populations of *G. rostochiensis* and *G. pallida* were detected (57 and 88 % *G. rostochiensis*, respectively).

As previously observed in 2000, inoculation treatments of *R. solani* caused a significant delay in emergence. However no interrelationships were found between *G. rostochiensis* initial population densities, inoculation treatments and the emergence of potato plants.

4.3.2.1 6 week harvest

As in field experiment 2000, *G. rostochiensis* infestations and stolons infected by *R. solani* were positively related (Figure 4.21). This relationship was only observed within plots inoculated with *R. solani* and was significant using measurements of both *G. rostochiensis* densities within the soil and the density of invading juveniles within potato root tissue (Table 4.8). By plotting data sets of nematode invasion and stolon canker from field experiments 2000 and 2001 (Figure 4.22), it was apparent that the measurements from the two years followed a similar trend. Linear regression analysis of the two data sets did not indicate any significant difference between the regression lines. Consequently a single regression line was used to describe the relationship found over the two years of field studies (Figure 4.22). The relationship found between the densities of *G. rostochiensis* within potato roots and stolon infections from the two years of field studies can be recognised as being moderate to strong (r^2 = 0.5536). This consistent positive trend strongly suggests that stolon infection by *R. solani* is

increased during the period of invasion and parasitic behaviour in the lifecycle of *G. rostochiensis*.

It was considered that stolon infections by *R. solani* might affect the degree of stolon branching and thus influence tuber size distribution and yield. Therefore, stolons were initially categorised as either primary, lateral or branched. However, since the majority of stolons formed were primary, it was not feasible to undertake statistical analyses and the stolon numbers were subsequently grouped as a whole.

A summary of the key relationships found between *G. rostochiensis* densities (Pi and juvenile invasion of potato roots) and diseases caused by *R. solani* is given in Table 4.8 for both inoculated plots and uninoculated plots. For inoculated plots, weak to moderate positive linear relationships were found (r^2 = 0.217-0.488) but no relationships were found in uninoculated plots. In inoculated plots, it can be seen that the strongest relationships occurred between initial population densities of *G. rostochiensis* and stolon infections or root canker score. Relationships between *G. rostochiensis* densities and the disease index of stem canker were found to be non-significant.

Multiple regression analyses conducted did not reveal any significant effects on potato growth measurements such as plant fresh/dry weight, stem numbers and tuber numbers weights.

Table 4.7 Potato cyst nematode Pi and species profile (determined by PCR) in plots either inoculated with *R. solani* or left uninoculated from field experiment 2001 (Swans Leasow Field, Harper Adams University College) presented in the order that the plots were paired together

	Inoculated plots			Uninoculated plots		
Block	Eggs g^{-1} soil	*% Globodera rostochiensis*	*% G. pallida*	Eggs g^{-1} soil	*% Globodera rostochiensis*	*% G. pallida*
1	10.7	≥95	0	15.8	≥95	0
1	14.6	≥95	0	14.8	≥95	0
2	18.6	≥95	0	13.1	≥95	0
2	22.1	≥95	0	23.8	≥95	0
3	26.2	≥95	0	24.0	≥95	0
3	29.8	≥95	0	31.9	57	43
4	20.5	≥95	0	20.7	≥95	0
4	22.2	≥95	0	25.2	≥95	0
5	43.5	≥95	0	41.0	≥95	0
5	70.0	≥95	0	59.4	≥95	0
6	51.3	≥95	0	56.9	≥95	0
6	58.1	≥95	0	58.0	≥95	0
7	32.4	≥95	0	37.5	≥95	0
7	40.4	≥95	0	37.9	88	12
8	30.3	≥95	0	23.9	≥95	0
8	61.4	≥95	0	60.4	≥95	0
9	48.8	≥95	0	48.1	≥95	0
9	71.2	≥95	0	69.2	≥95	0
10	28.3	≥95	0	30.9	≥95	0
10	37.2	≥95	0	41.2	≥95	0
11	86.4	≥95	0	79.6	≥95	0
11	91.9	≥95	0	107.9	≥95	0
12	63.5	≥95	0	63.6	≥95	0
12	66.0	≥95	0	67.0	≥95	0

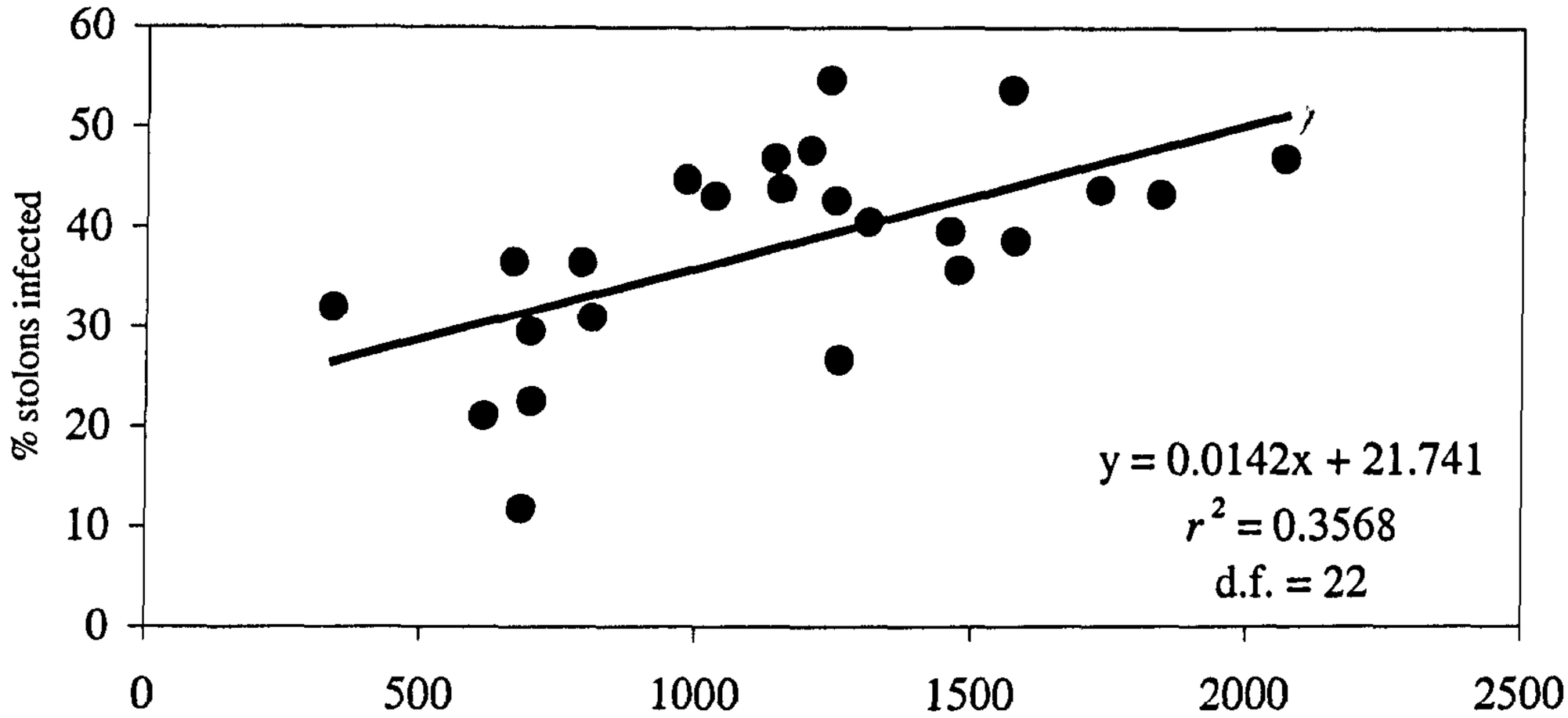


Figure 4.21 The relationship between invasion of potato (cv. Désirée) roots by *G. rostochiensis* juveniles and infection of stolons by *R. solani* in plots inoculated with *R. solani*, 6 weeks after planting field experiment 2001

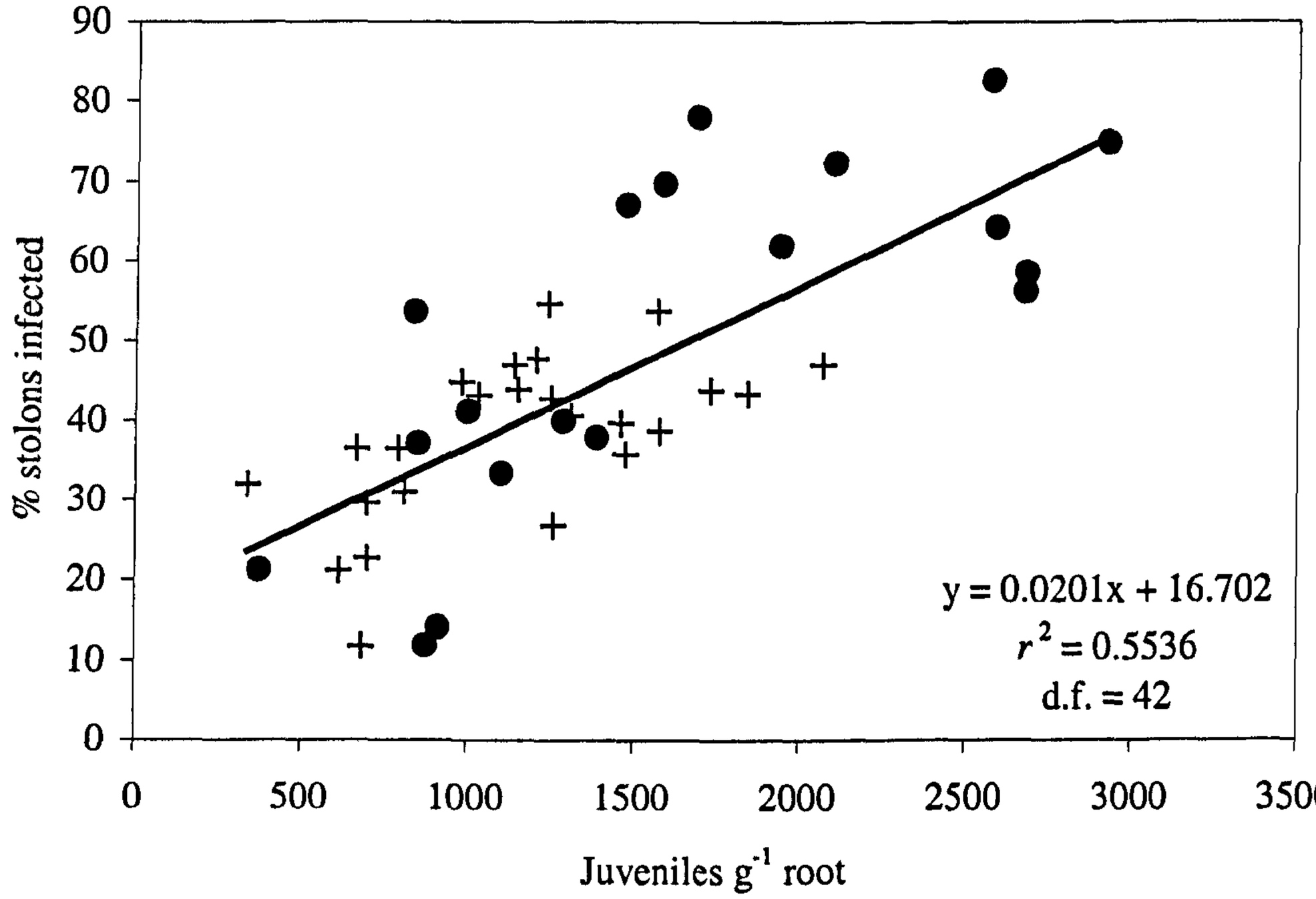


Figure 4.22 The relationship between invasion of potato (cv. Désirée) roots by *G. rostochiensis* juveniles and infection of stolons by *R. solani* in inoculated plots, 6 weeks after planting field experiments 2000 (●) and 2001 (+)

Table 4.8 The relationships observed between densities of *G. rostochiensis* (within soil and potato roots) and *R. solani* diseases on different parts of potatoes (cv. Désirée) 6 weeks after planting field plots either (a) inoculated with *R. solani* or (b) left uninoculated in 2001

(a) Inoculated plots

Explanatory Variate	Response Variate	Significance (*P*)	Standard error (S.E.)	Coefficient of determination (r^2)
Juvenile g^{-1} root	% stolons infected	0.002	0.00408	0.328
Pi (eggs g^{-1} soil)	% stolons infected	<0.001	0.0762	0.488
Juvenile g^{-1} root	% main stems infected	0.013	0.00282	0.217
Pi (eggs g^{-1} soil)	% main stems infected	0.051	0.0564	0.124
Juvenile g^{-1} root	Root canker score	0.008	0.00022	0.248
Pi (eggs g^{-1} soil)	Root canker score	0.002	0.00389	0.338

d.f. = 22

(b) Uninoculated plots

Explanatory Variate	Response Variate	Significance (*P*)	Standard error (S.E.)	Coefficient of determination (r^2)
Juvenile g^{-1} root	% stolons infected	0.097	0.00613	0.08
Pi (eggs g^{-1} soil)	% stolons infected	0.214	0.0776	0.027
Juvenile g^{-1} root	% main stems infected	0.063	0.0184	0.11
Pi (eggs g^{-1} soil)	% main stems infected	0.147	0.234	0.052
Juvenile g^{-1} root	Root canker score	0.634	0.00029	0
Pi (eggs g^{-1} soil)	Root canker score	0.646	0.00351	0

d.f. = 22

4.3.2.2 Final harvest (yield)

The compact plot design of this experiment was developed with the aim of optimising comparisons between the six-week harvest assessments with final yield and the development of black scurf disease on the progeny tubers. However, correlation and linear regression analyses undertaken on *G. rostochiensis* population densities and measurements of black scurf did not reveal any significant relationships.

Figure 4.23 shows the relationship between densities of juvenile *G. rostochiensis* within the roots of 6 week old plants and final tuber yield (in tonnes/hectare) of neighbouring plants in plots either inoculated or uninoculated with *R. solani*. Plots inoculated with *R. solani* produced significantly lower tuber yields than those left uninoculated ($P<0.001$) (Figure 4.23). In addition, weak negative relationships were found between *G. rostochiensis* juvenile invasion of the roots of 6-week-old plants and tuber yield all plots ($P<0.05$). Multiple regression analysis revealed that the effect of *G. rostochiensis* root invasion upon stolon infection by *R. solani* had a significant effect on final tuber yield in both inoculated ($P<0.024$) and uninoculated plots ($P<0.017$). The proportions of tubers belonging to 10 mm size brackets (grade) were not found to be related to either *R. solani* diseases or densities of *G. rostochiensis*.

When *G. rostochiensis* initial population densities (Pi) are plotted against the multiplication rate (Pf/Pi), a negative double exponential curve is obtained (Evans & Stone, 1977). Observations from this experiment indicated that the multiplication rate was additionally affected by inoculation treatments of *R. solani* (Figure 4.24). Comparison of the two lines between the two inoculation treatments revealed a significant difference ($P<0.005$).

Furthermore, a negative relationship was found between measurements of stolon infections caused by *R. solani* (6-week harvest) and multiplication rate in inoculated plots ($P<0.002$, $r^2 = 0.325$). Unlike field experiment 2000, linear regression analysis of *G. rostochiensis* juvenile densities and multiplication rate was not significant ($P>0.05$). In uninoculated plots, no relationship was found between stolon infections and multiplication rate ($P>0.05$), however, a weak negative relationship was found between *G. rostochiensis* juvenile densities and the multiplication rate ($P<0.002$). The results of these regression analyses are summarised in Table 4.9.

Table 4.9 Simple linear regression analysis showing the response of *G. rostochiensis* multiplication to invasion of potato (cv. Désirée) roots by *G. rostochiensis* juveniles or the incidence of stolons infected by *R. solani*, at 6 weeks after planting field experiment 2001

Treatment	Explanatory variates	Significance (P)	Standard error (S.E.)	Coefficient of determination (r^2)
Inoculated plots (d.f = 22)	% Stolons infected	0.002	0.281	0.325
	Juveniles g^{-1} root	0.062	0.0077	0.011
Uninoculated plots (d.f = 22)	% Stolons infected	0.104	0.807	0.076
	Juveniles g^{-1} root	0.003	0.0214	0.309

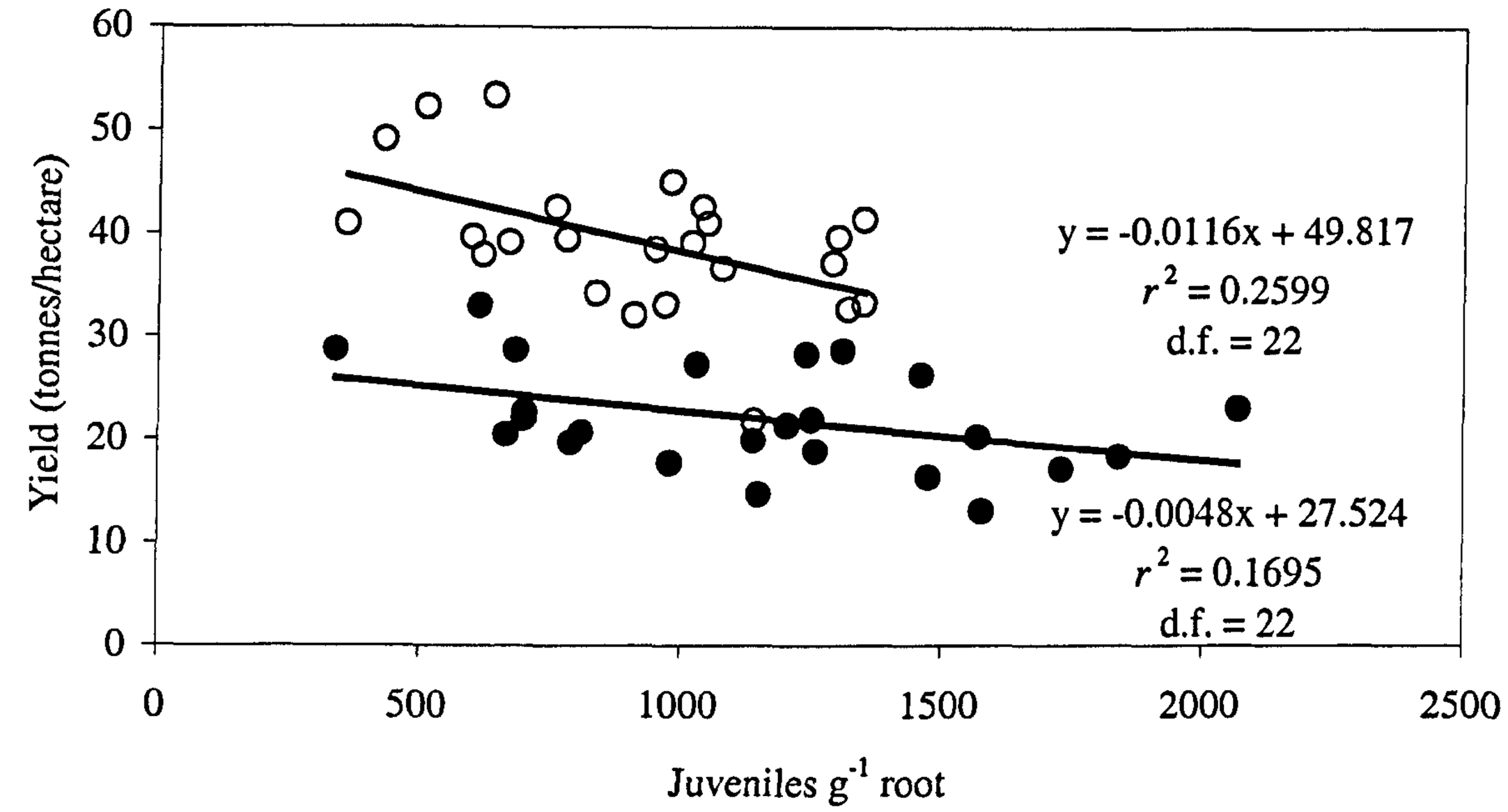


Figure 4.23 The relationship between invasion of potato (cv. Désirée) roots by *G. rostochiensis* (six weeks after planting) and final tuber yield in field plots either inoculated with *R. solani* (●) or left uninoculated (○) during 2001

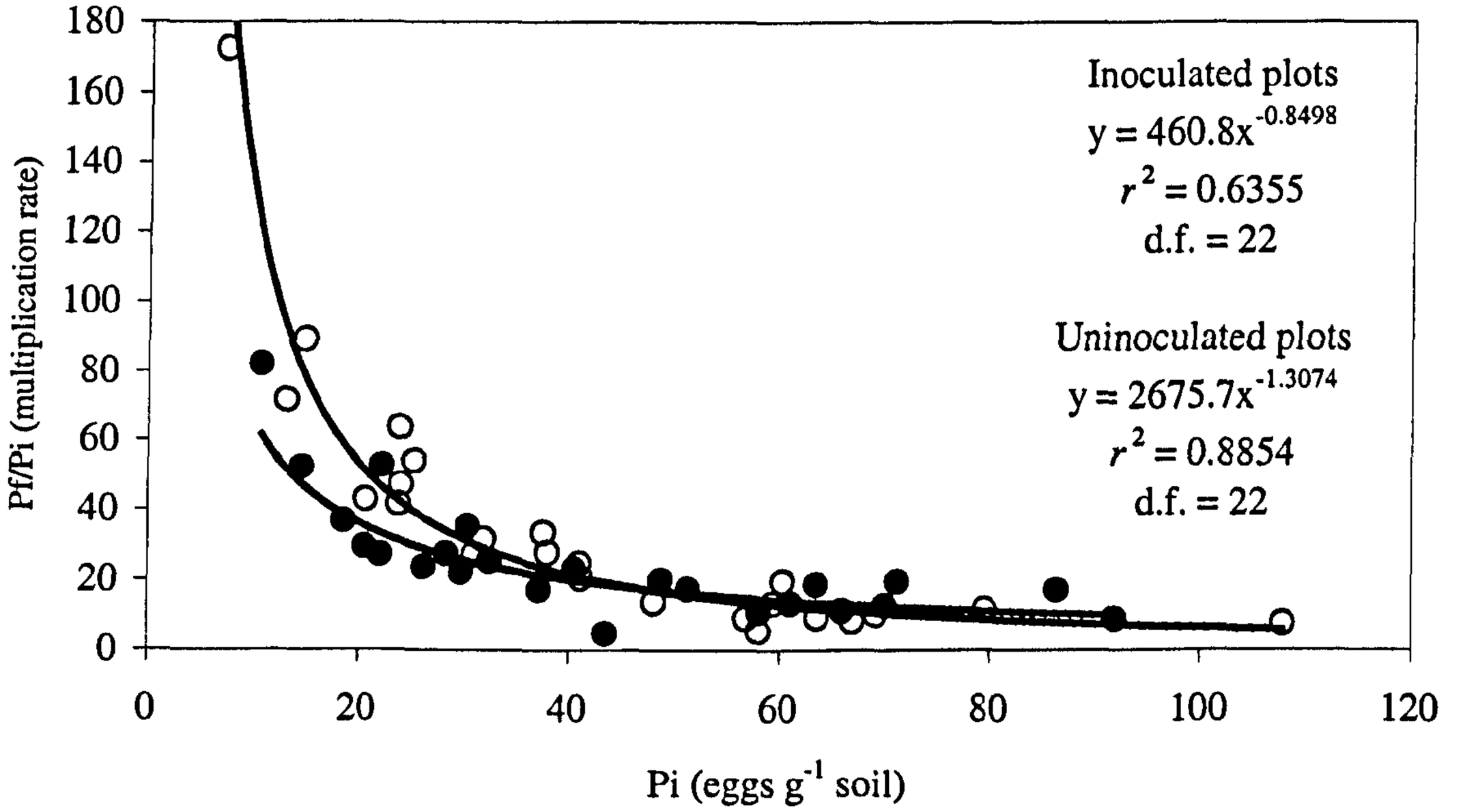


Figure 4.24 The relationship between initial population densities (Pi) and multiplication rate of *G. rostochiensis* in field plots either inoculated with *R. solani* (●) or left uninoculated (○) during 2001

4.4 Discussion

The results of both years of field studies have indicated a synergistic interaction between the potato cyst nematode *Globodera rostochiensis* and the fungal pathogen *Rhizoctonia solani* in potatoes. Positive relationships were consistently found between *G. rostochiensis* densities and *R. solani* infection of various potato parts, but particularly between the invasion of potato roots by *G. rostochiensis* juveniles and the incidence of stolons infected by *R. solani*. This relationship is the key finding of this work and has not been reported elsewhere. The closest record to these findings are those of Mazurkiewicz-Zapalowicz & Waker-Wójciuk (1994) where severe necroses of potato are described during combined infestations of *G. rostochiensis* and *R. solani* in comparison to treatments of either organism alone. Unfortunately, these authors did not specify which region of the plant was affected by the pathogen or the assessment methods used. This information would have been useful to compare with the current study, which has given an indication that *G. rostochiensis* infestations influence regional infections of *R. solani* infection, in particular stolon canker.

As discussed in Chapter 1 and a recently published review (Back *et al.*, 2002), disease complexes involving plant parasitic nematodes and soil-borne fungi may result via a range of mechanisms. The observations found during this experimental work suggest that interactions between *G. rostochiensis* and *R. solani* could be indirect. This hypothesis is based upon the findings that *G. rostochiensis* and *R. solani* infection sites occur on different subterranean regions of the host, whereby *G. rostochiensis* has its lifecycle within the roots of potato (Chapter 1), whilst associated *R. solani* infections have most frequently been found on the stolons. The stolons and roots of the potato plant grow in relativity close proximity to one another since both originate from shared nodes on the subterranean stem. This spatial

arrangement may be important because plant parasitic nematodes are hypothesised to cause modifications in the rhizosphere, which increase the attraction of pathogenic fungi to their host plants (Golden & Van Gundy, 1972). To elaborate further, a number of authors (Bergeson, 1972; Van Gundy *et al.*, 1977) assert that nematode damage or inhabitation of root tissue modifies the constituents of root exudates. Root exudates from nematode infested plants have been found to have higher concentrations of nutrients such as carbon and nitrogen (Van Gundy *et al.*, 1977), which are recognised as essential nutrients to fungi such as *R. solani* (Weinhold *et al.*, 1972). Contrary to the root exudate hypothesis, it could be argued that nematode induced physiological changes (Chapter 1, section 1.3.2.2, page 34) or resistance breaking (Chapter 1, section 1.3.2.4, page 38) may have enhanced the *R. solani* infections. However, an increase in overall disease symptoms might be expected if either of these mechanisms were in operation. There may be other explanations for the relationships observed, such as *R. solani* utilising nematode induced wounds or feeding sites. Results from 2001 indicated a weak relationship between the invasion of potato roots by *G. rostochiensis* juveniles and the incidence of root cankers caused by *R. solani*. Nonetheless, the mechanism concerning nematode wounding is unlikely to account for the increase in stolon canker observed.

During 2000, a number of harvest dates (4, 6 and 8 weeks after planting) were used to monitor the progress of *G. rostochiensis* and *R. solani*. The strongest relationship between *G. rostochiensis* densities within potato roots and the percentage of stolons infected was at 6 weeks after planting. On this basis the 2001 field study was conducted with a single harvest date, 6 weeks after planting. While this observation may be coincidental, the increase in *G. rostochiensis* juvenile stages J4-J5 (see Table 4.3, page 121) at six weeks after planting may correspond to the stronger relationships with *R. solani* infections. At juvenile stages J3- J5, *G. rostochiensis* remain sedentary (Evans & Stone, 1977) while taking nourishment from the

induced syncytia (see Chapter 1, section 1.2.4.3, page 27). Bearing in mind the hypotheses already highlighted for synergistic nematode-fungus interactions, it is possible that an increased number of cells with high metabolic activity (syncytia) could have either enriched the root exudate constituents or improved the nutritional properties of the root tissue to consequently promote the activity of *R. solani*. A similar view has also been expressed by Taylor (1990), who suggested that the 3-4 week pre-inoculation treatments used in experiments investigating root-knot nematodes were critical to the severity of fungal diseases (Wajid Khan & Muller, 1982; Negrón & Acosta, 1989) because of the syncytial development at this time.

At 8 weeks after planting, the relationship between *G. rostochiensis* and stolon infections is less well defined. This is most probably due to the lower densities of *G. rostochiensis* (see Table 4.3, page 121), which relate to the proportion of nematodes leaving the roots at this time (Evans, 1969). Work by Evans (1969) explored the densities of *Heterodera rostochiensis* (syn. *Globodera rostochiensis*) over the cropping season of the potato cv. Majestic. Results from his research are similar to those reported here, although nematode populations appeared to peak slightly later, at 6-8 weeks after planting. This variation is likely to be the result of contrasting experimental parameters such as the cultivar used, physiological age and the *R. solani* treatment used in the current work. Evans (1969) showed that after the main peak of juvenile densities (8 weeks after planting) within the roots, numbers initially decreased rapidly before slowly increasing again due to invasion of juveniles from the second generation 9-10 weeks after planting.

In both field studies, there was very little evidence of a relationship between *G. rostochiensis* densities within the soil or plants sampled and black scurf on the daughter tubers.

Furthermore, weak or non-significant relationships were found between symptoms of stem and stolon canker and black scurf. In published literature, opinions are divided on whether early disease symptoms caused by *R. solani* such as stem and stolon canker relate to the later development of black scurf on daughter tubers. Hide *et al.* (1989a) found little consistency between stem canker symptoms and black scurf on the progeny tubers of five early and seven main crop cultivars in two years of field experiments. Similarly, Simons & Gilligan (1997a) found good relationships between the amount of *R. solani* seed-borne inoculum and stem and stolon canker, although correlations between stem canker and black scurf, however, were far weaker, suggesting that soil-borne inoculum of *R. solani* was involved. In the present work, a direct relationship between black scurf and *G. rostochiensis* densities was not considered likely. Instead it was hypothesised that synergistic interactions between *R. solani* and *G. rostochiensis* could result in increased stem and stolon canker symptoms that in turn increased black scurf on the daughter tubers.

In 2000, a relationship was found between densities of *G. rostochiensis* within the soil (Pi) and % emergence of plants in plots inoculated with *R. solani.* In addition, a strong negative relationship was observed between Pi and the % daily increase in ground cover occupied by the potato canopy in *R. solani* inoculated and uninoculated plots. Whilst inoculation with *R. solani* can also delay emergence (Banville, 1989; Jeger *et al.*, 1996, Secor & Gudmestad, 1999), the combined presence of these organisms may further extend the period taken for plants to emerge as indicated by Figure 4.6 (page 110). A further delay in emergence may be critical since previous research has shown that stem canker symptoms increase very little (Hide *et al.*, 1985a, 1989a) or even decrease (Simons & Gilligan, 1997a) after plant emergence. Van Emden (1965) hypothesised that potato stems gained resistance to stem canker following exposure to light, which causes a switch to autotrophic nutrition. It should

also be noted that the intensity and wavelength of light is known to increase the production of potato glycoalkaloids (Gull & Isenbreg, 1960), which have been shown to have anti-fungal activity against a number of plant pathogens (Fewell & Roddick, 1993) and have a systemic distribution in the potato plant (Friedman & Dao, 1992). Moreover, Fewell *et al.* (1994) found that a 1:1 ratio of the glycoalkaloids, alpha-solanine and alpha-chaconine (concentration = 50μM) were able to significantly inhibit the growth of *R. solani*. Ultimately, the prolonged period of time taken for plants to emerge during exposure to concomitant infestations of *G. rostochiensis* and *R. solani* may accentuate the symptoms of stem and stolon canker.

It is well established that infestations of potato cyst nematodes *Globodera rostochiensis* and *G. pallida* adversely affect the yield of potato (Evans & Stone, 1977; Brodie *et al.*, 1993; Haydock & Evans, 1998). Correspondingly, the results from the present work revealed a negative relationship between *G. rostochiensis* infestations and tuber yield in both *R. solani* inoculated and uninoculated plots, although in the second field experiment (2001) such relationships were far weaker.

In contrast to potato cyst nematodes, the effect of *R. solani* causing stem and stolon canker on potato yield is more complex. The majority of authors do not report a loss in total yield following stem and stolon canker but rather a shift in the distribution pattern of tuber sizes (Hide *et al.*, 1985b, 1989b; Banville, 1989; Simons & Gilligan, 1997b). For example, Simons & Gilligan (1997b) observed a significant increase in baker (60-80mm) and oversized (>80mm) tubers with a corresponding decrease in main sized tubers (40-60mm) in potato (cv. Estima) plots inoculated with *R. solani*. Read *et al.* (1989) suggested that stolon pruning is the probable cause of increased oversized tuber fractions due to the remaining tubers becoming principal nutrient sinks. Potato cultivar and the time of harvesting are also critical to the

overall effects of stem and stolon canker on the tubers produced (Hide *et al*, 1985b, 1989b). Main-crop cultivars such as Désirée are less prone to yield loss (Hide *et al.*, 1989b; Read *et al.*, 1989) since the longer growing season allows more time for plants to compensate for shoot and stolon damage, whereas early harvested crops (harvested in June-July) can suffer from depressed yields (Hide *et al.*, 1985b).

Unlike previous research (Hide *et al.*, 1985b, 1989b; Simons & Gilligan, 1997b), there were no observed relationships between disease severity (stolons infected) and tuber size fractions in either of the field experiments. This disparity may be a result of the combined presence of *G. rostochiensis* and *R. solani*. The increased proportion of baker or oversized tubers previously observed in other work investigating *R. solani* (Hide *et al.*, 1985b, 1989b; Simons & Gilligan, 1997b) may have been masked by the influence of *G. rostochiensis* infestations, which may increase the proportion of smaller tubers produced as indicated by Grove (1999).

During the 2000 field trial, no significant differences were found between tuber yield for *R. solani* inoculated and uninoculated plots, although comparisons between these two treatments are questionable because of the high incidence of *R. solani* disease symptoms found in uninoculated plots (100% incidence of stem and stolon canker). Results from 2000, indicated that *R. solani* was the principal cause of yield loss in inoculated plots. However, in plots left uninoculated, both *G. rostochiensis* densities and stolon infections had significant negative relationships with tuber yield. The results from the second field experiment (2001) were in contrast to those found in the first (2000). For example, significant differences were found between regression lines obtained for *G. rostochiensis* densities against tuber yield from inoculated and uninoculated plots, where yield was only significantly reduced by the inoculation treatment. This might be explained by the comparatively lower incidence of *R.*

solani infections found within the uninoculated plots of this experiment (58% incidence of stem and stolon canker). Although weak negative trends were seen between stolon canker and tuber yield, these were not significantly different to those seen in the first experiment (2000).

The effect of both *G. rostochiensis* and *R. solani* on yield is difficult to interpret. One method is to use multiple regression analysis to test the effects of several explanatory variates such as the number of *G. rostochiensis* juveniles per g root and % stolons infected on a response variate such as yield. This method revealed that yield was significantly affected by the combined presence of *G. rostochiensis* and *R. solani* in 2001 but not in 2000. The results from 2000 are surprising since *G. rostochiensis* densities within potato roots formed a positive relationship with % stolons infected, whilst stolon infections were negatively related to tuber yield. It could be argued that the stolon infections responsible for yield depression are a reflection of the nematode densities.

An alternative approach to modelling yield reduction in response to independent and combination treatments of fungi and nematodes within field experiments is to use a fumigant or nematicide treatment to reduce the nematode populations. For example, Golden & Van Gundy (1975) adopted this type of approach during field investigations on interrelationships between the root-knot nematode *Meloidogyne incognita* and *R. solani* on okra and tomato. In their experiment, a field site with high densities of *M. incognita* and *R. solani* was split into 24 plots with 3 treatments: - 1. Fumigation with methyl bromide (to manage micro-flora and fauna), 2. Fumigation with ethylene dibromide (to reduce root-knot nematodes) or 3. Left untreated. This experimental design has the advantage of potentially teasing apart the individual and combined effects of nematode and pathogen upon plant yield. A particular weakness of this type of approach, however, is the assumption of an even spatial distribution

of nematode and fungal population densities, which are generally recognised to have patchy distributions (Boag *et al.*, 2000; Gilligan *et al.*, 1996). Furthermore, fumigants or nematicides used to manipulate experimental treatments may have adverse effects upon the pathogen (Ruppel & Hecker, 1982; Scholte, 1987) or on other non-target micro-flora and fauna affecting to the disease complex (Hofman *et al.*, 1991; Lootsma & Scholte, 1997; Kerry, 2000).

The effect of combined infestations of *G. rostochiensis* and *R. solani* on potato yield has been reported elsewhere. In glasshouse work by Grainger & Clark (1963, 1965), significantly lower tuber yields (cv. Epicure) were found following exposure to combined infestations of *G. rostochiensis* and *R. solani* in comparison to treatments with either organism alone. Contrary to these findings, Mazurkiewicz-Zapalowicz & Waker-Wójciuk (1994) found no differences between the yield of glasshouse grown potatoes (cv. Mila) treated with combined and independent treatments of *G. rostochiensis* and *R. solani*. The conflicting results of these two studies are difficult to compare because of the different potato cultivars used and the lack of information regarding prevailing environmental conditions and soil type.

The findings discussed thus far have focussed on relationships between *G. rostochiensis* and *R. solani* in terms of disease development and yield suppression. However, both field studies also indicate potential antagonism between *R. solani* and *G. rostochiensis*. For example, during 2000, weak relationships were found between the percentage of stolons infected by *R. solani* or total *G. rostochiensis* juveniles per gram of potato root and the proportion of *G. rostochiensis* juveniles developing into females, 8 weeks after planting. These relationships were investigated, since previous research by other workers (Davide & Trianlaphyllou, 1967; Trudgill, 1967) has indicated that increasing densities of *G. rostochiensis* within potato roots

can result in an increase in the ratio of males to females. Furthermore, Ketudat (1969) observed a decrease in the proportion of female *Heterodera* (syn. *Globodera*) *rostochiensis* juveniles on tomato plants co-infected with either *R. solani* or *Verticillium albo-atrum* compared to plants which were infested with *H. rostochiensis* alone. Although the mechanisms behind these observations are less clear, Trudgill (1967) suggested that plants invaded by higher densities of *G. rostochiensis* might have less available nutrients to individual nematodes, which in turn could affect sex determination. This hypothesis seems feasible for both higher densities of nematodes and situations where two or more pathogens are present and competing for nutrients. It is also worth highlighting the findings of Fatah & Webster (1983), who found that the feeding sites (giant cells) of the root knot nematode *Meloidogyne incognita* were disfigured and retarded during concomitant infections by *Fusarium oxysporum* f.sp. *lycopersici*. Such observations could correspond to the effect of *R. solani* infections on the syncytia of *G. rostochiensis*, which ultimately could affect sex determination or the development of female nematodes.

Results from the 8-week harvest during 2000 have indicated that sex determination may be altered during higher infections of *G. rostochiensis* or with increasing severities of stolon canker, although such relationships were weak. In addition, when multiple regression analysis was undertaken to determine whether the shift in male/female ratio was altered by stolon canker incidence, density of *G. rostochiensis* within the potato roots or both, or an interaction of the two explanatory variates, no significant effects were observed. Therefore, these results should be treated with caution and further experiments would need to be designed to explore this hypothesis in greater detail.

In both 2000 and 2001, negative linear relationships were found between mean stolon infections caused by *R. solani* and the multiplication rate of *G. rostochiensis*, suggesting that potato plants heavily infected with *R. solani* will have a lower number of female nematodes maturing into cysts. Similarly, the density of *G. rostochiensis* juveniles found in potato roots 4 –8 weeks after planting was also negatively related to the multiplication rate of *G. rostochiensis*. This, however, was not unexpected since multiplication rate is recognised to decrease with increasing Pi of *G. rostochiensis* (Evans & Stone, 1977). Given that a positive relationship was found between *G. rostochiensis* invasion of potato roots and the incidence of stolon infections caused by *R. solani*, it might be argued that the co-linearity between these variates may be reflected in relationships with the multiplication rate of *G. rostochiensis*. However, results of multiple regression analyses have shown that both densities of *G. rostochiensis* juveniles found in potato roots and the incidence of stolon infections caused by *R. solani* were related to the multiplication rate of *G. rostochiensis* in plots inoculated with *R. solani* during 2000. These results are further supported by results from 2001, which indicated a lower multiplication rate in plots inoculated with *R. solani* compared to plots left uninoculated. Consequently, inoculation treatment with *R. solani* may be responsible for disrupting the development of females or altering sex determination to subsequently suppress the multiplication rate of *G. rostochiensis*, the mechanisms for which were discussed earlier in this section. These results support the previous findings from field experiment 2000 where a weak relationship was found between the male to female ratio of *G. rostochiensis* and stolons infected with *R. solani*. The fact that no relationships were found between stolons infected by *R. solani* and the multiplication rate of *G. rostochiensis* in uninoculated plots in both trials is not surprising given the unpredictable patchiness of naturally occurring soil-borne inoculum of *R. solani* (Jager & Velvis, 1989). Relationships occurring between the densities of juvenile nematodes found in the roots and the multiplication rate of *G. rostochiensis* is consistent with

well established knowledge that increasing populations of potato cyst nematodes result in a decrease in their multiplication rate most probably because of competition for nutrients and subsequent changes in sex determination.

In comparison to the glasshouse work of this project (Chapter 3), the field experiments have provided considerably more detailed and convincing evidence of synergistic interactions between *G. rostochiensis* and *R. solani*. No significant relationships were found between *G. rostochiensis* infestations and *R. solani* diseases in either of the glasshouse experiments. This may be due to the lower densities of *G. rostochiensis* juveniles invading potato roots in the glasshouse work. Moreover, the range of *G. rostochiensis* juvenile densities recovered from potato roots in field experiments was considerably wider than those recorded during glasshouse experiments (Figure 4.25). This suggests that the wider range of *G. rostochiensis* densities present in the field experiments allowed the relationship between *G. rostochiensis* densities and *R. solani* diseases on potatoes to be observed more clearly. Sikora & Carter (1987) reported that only 6 out of 39 (15%) studies investigating nematode-pathogen disease complexes between 1976 and 1986 involved field based experiments. Considering the findings from the current work, future investigations on disease complexes involving nematodes and fungi would benefit from the inclusion of field based studies. Field experiments, have the additional advantage of exploring interactions in their natural environment where potentially important biotic and abiotic factors are not excluded.

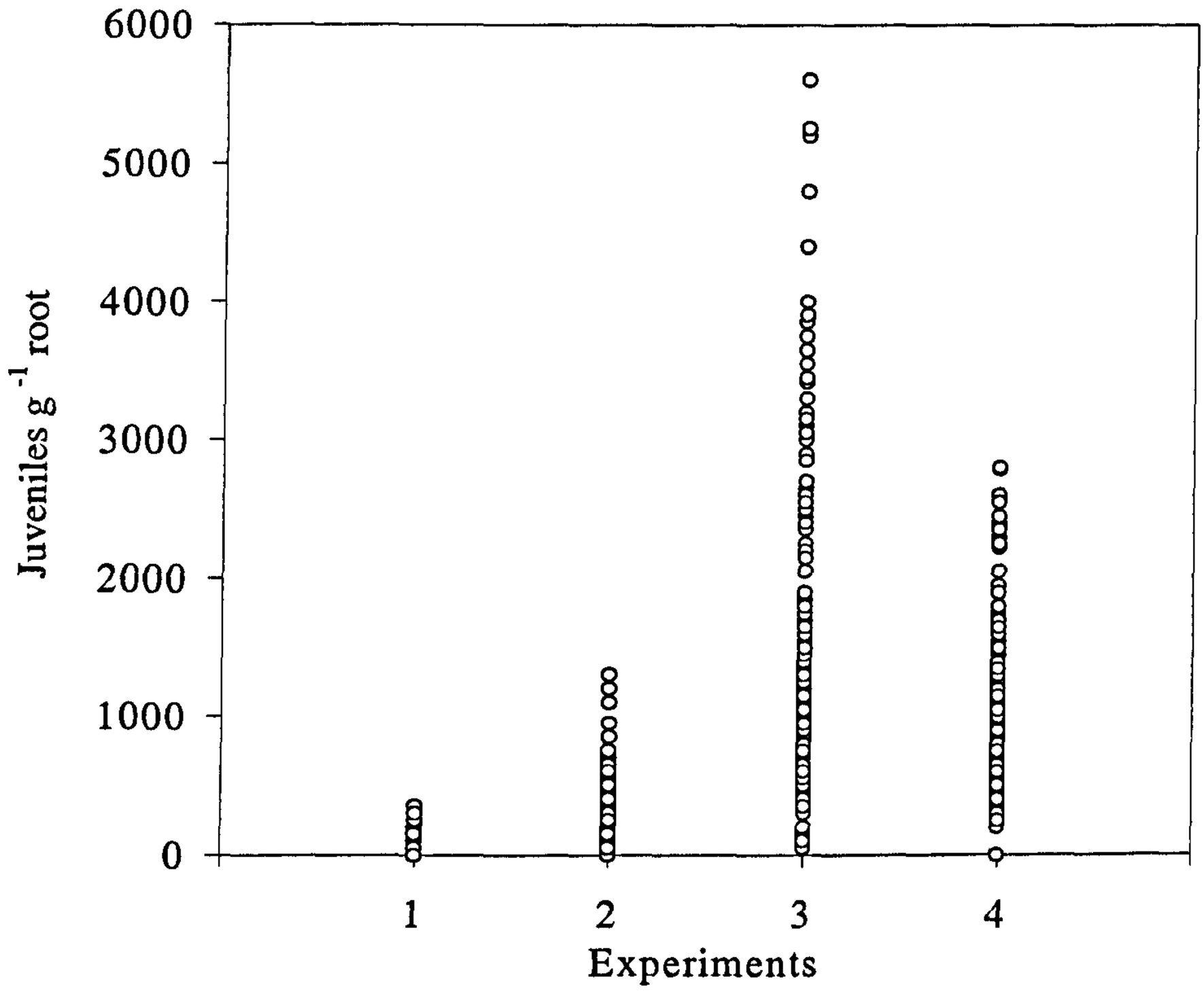


Figure 4.25 A graphical representation of the range of *G. rostochiensis* juveniles found to invade the roots of potatoes in 1) Glasshouse experiment 2000, 2) Glasshouse experiment 2001, 3) Field experiment 2000 and 4) Field experiment 2001, six weeks after planting

In general, the majority of findings from these two experiments have related to measurements of *G. rostochiensis* juvenile densities found in potato roots and the proportion of stolons infected with *R. solani*. The estimation of juvenile *G. rostochiensis* nematodes from root tissue appears to be a reliable measurement to compare against the development of *R. solani* diseases as well as other plant growth response measurements. In contrast, measurements of *G. rostochiensis* from soil seem less appropriate since there is no measurement of how viable the eggs (J1) within the cysts are, although it is possible to estimate viability through staining methods such as Meldola's blue (Shepherd, 1986). The stem canker key designed to measure the severity of *R. solani* symptoms was generally not found to produce very good relationships with response variates in comparison to measurements of stolons infected or stems pruned. In view of the fact that the key incorporates all symptoms of stem and stolon canker, there could be a loss of sensitivity in using such assessment methods. Simons & Gilligan (1997a) also report that their assessment key for *R. solani* did not add any extra information to assessments of presence and absence of infection.

A potentially limiting factor of the two studies was the lack of information concerning the quantity of *R. solani* soil-borne inoculum present in the fields prior to planting. It was decided that it would be unfeasible to attempt to estimate soil-borne inoculum for several reasons. *Rhizoctonia solani* is the anamorph of *Thanatephorus cucumeris* and consequently does not produce sexual spores. Instead the fungus exists as mycelium and sclerotia within the soil, which makes the estimation of disease causing propagules extremely difficult. The first problem is the isolation and identification of the fungus, which for many years was achieved by lengthy culturing of the fungus from soil pellets (Henis *et al.*, 1978) on selective media (Ko & Hora, 1971; Castro *et al.*, 1988). Recently, Lees *et al.* (2002) developed a quantitative 'real time' PCR (polymerase chain reaction) assay for the detection and quantification of *R. solani*

anastomosis group 3 (AG3) from soil. Although, this molecular tool successfully detected *R. solani* AG3 from soil the results were inconsistent, which the authors attribute to PCR inhibitory compounds emanating from soil. Further tests using *Chenopodium quinoa* seeds to bait *R. solani* from soil samples (Thornton *et al.*, 1999), improved the extraction *R. solani* AG3 DNA. However, this extra step could produce numerous areas for variation to occur. For example, different soil types (from different soil samples) are likely to affect the attraction and growth of the fungus to the seeds. Regardless of the detection methods, there is still a large proportion of work required on determining the most appropriate method for sampling the fungus. The development of a reliable detection system with a realistic strategy for sampling would be invaluable to further studies on *R. solani* and would help provide fundamental information to potato growers.

The results of these experiments have demonstrated that by using relatively simple measurements and statistical analyses, relationship patterns could be observed between *G. rostochiensis*, *R. solani* and their mutual host the potato. The consistency of relationships between *G. rostochiensis* densities and the development of *R. solani* diseases, particularly stolon canker, has given good evidence that a synergistic interaction occurs between the two organisms. In the subsequent chapter investigations exploring the mode of this interaction are reported. It has also been shown that while *R. solani* might benefit from infestations of *G. rostochiensis*, nematode development may be impaired during concomitant infections with *R. solani*

CHAPTER 5.0 – CONTROLLED ENVIRONMENT EXPERIMENTS INVESTIGATING MODIFICATIONS IN THE POTATO RHIZOSPHERE DURING INFESTIONS OF *GLOBODERA ROSTOCHIENSIS* AND SUBSEQUENT EFFECTS ON THE GROWTH OF *RHIZOCTONIA SOLANI*

CHAPTER 5.0 – CONTROLLED ENVIRONMENT EXPERIMENTS INVESTIGATING MODIFICATIONS IN THE POTATO RHIZOSPHERE DURING INFESTIONS OF *GLOBODERA ROSTOCHIENSIS* AND SUBSEQUENT EFFECTS ON THE GROWTH OF *RHIZOCTONIA SOLANI*

5.1 Introduction

Two years of field experiments consistently showed a clear relationship between invasion of potato roots by *Globodera rostochiensis* and the infection of stolons by the fungal pathogen *Rhizoctonia solani*. In Chapter 4, the possible mechanisms behind this relationship were discussed and it was hypothesised that damage or colonisation of potato roots by *G. rostochiensis* might result in quantitative or qualitative changes in the release of root exudates, which could subsequently influence the growth and attraction of *R. solani*.

Root exudates can broadly be described as substances that are released from root surfaces other than water (Hale *et al.*, 1971). The most common constituents of root exudates include mucilage (a gelatinous material predominantly comprised of polysaccharides), ectoenzymes, sloughed cells, organic acids, sugars, phenolics, vitamins and amino acids (Rovira, 1965; Hale *et al.*, 1971; Nelson, 1990; Marschner, 1997). Many of these exuded substances provide sources of nutrition for microorganisms existing within the subterranean environment (Rovira, 1965; Nelson 1990; Griffiths *et al.*, 1999) including fungal pathogens such as *R. solani* (Reddy, 1980).

By providing a source of nutrition, root exudates may influence the attraction and growth of *R. solani* (Kerr, 1956; Martinson, 1965; Reddy 1980). In particular, positive relationships have been found between exudate constituents such as amino acids (aspartic acid, asparagine, histidine) and sugars (glucose and sucrose) and the pathogenicity and *in-vitro* mycelial growth

of *R. solani* (Reddy, 1980, Ritz *et al.*, 1996). This is well illustrated in the experiments of Weinhold *et al.* (1969, 1972) who found that the severity of lesions caused by *R. solani* on cotton plants (*Gossypium hirsutum*) could be increased if *R. solani* inoculum was formerly grown on a basal medium (glucose, KH_2PO_4, $MgSO_4.7H_2O$) containing the amino acid L-asparagine. However, when the *R. solani* inoculum was grown with lower concentrations of L-asparagine, lesion severity was reduced.

Plants infested with sedentary endoparasitic nematodes such as *G. rostochiensis* may produce higher quantities of root exudate constituents that are favourable to the growth and pathogenicity of fungal pathogens. For example, Van Gundy *et al.* (1977) attributed increases in the severity of *R. solani* root rot of tomato during concomitant infections with root-knot nematodes (*Meloidogyne incognita*) to a shift in the concentrations of carbohydrates and nitrogenous compounds within the root exudates of nematode infested plants. During the life cycle of *G. rostochiensis* (see Chapter 1, page 22) there are a number of stages where the profile or volume of root exudates could be modified (Figure 5.1). This chapter reports the findings of two experiments designed to examine temporal changes occurring in the root exudation of potato plants infested or uninfested with *G. rostochiensis* in relation to the growth of *R. solani.*

The aims of this work were to (i) amend media with root leachates collected from *G. rostochiensis* infested and uninfested potato plants at different times after infestation and record the growth of *R. solani* and (ii) measure carbohydrate and nitrogen content from the root leachates collected. The experiments employed in this work tested the null hypothesis that root leachates from potato plants infested with *G. rostochiensis* do not affect the growth of *R. solani.*

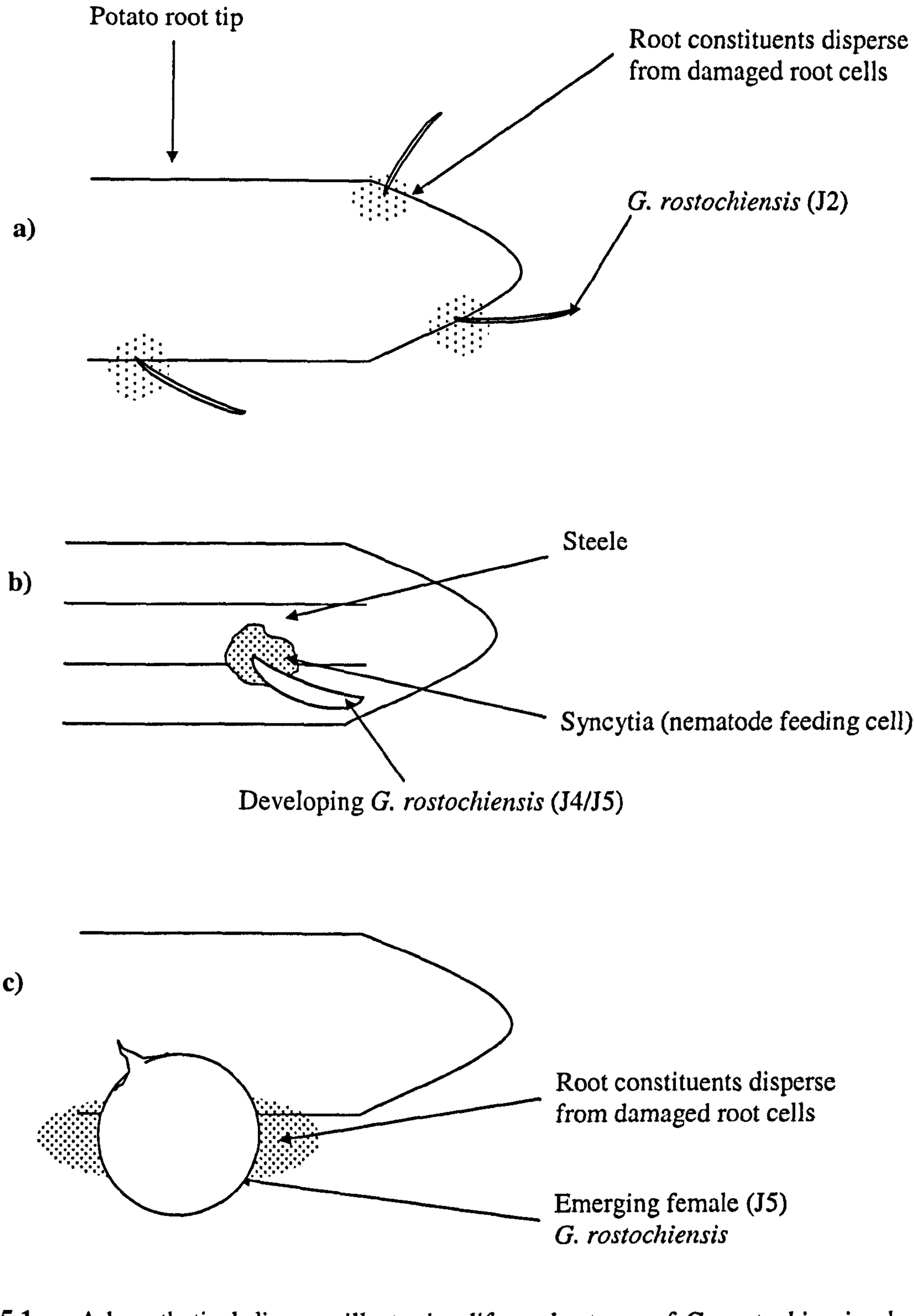


Figure 5.1 A hypothetical diagram illustrating life cycle stages of *G. rostochiensis* where potato root exudation may be modified: a) Damage to cortical cells during nematode penetration and invasion, b) Increased release of exudates constituents from potato roots with nematode feeding cells, c) Extensive damage to the roots cortex during the emergence of female nematodes

5.2 Materials and Methods

5.2.1 Experiment 1

5.2.1.1 Root leachate collection

The initial step of this experiment was to collect root leachates from potato plants either infested or uninfested with *G. rostochiensis*. The potato cultivar Désirée (class SE2) was selected for continuity between the current experiment and previous field experiments. Prior to planting, 60 tubers were chitted in a glasshouse (ca. 15-20 °C) until 3 mm sprouts had developed. Following chitting, a 'melon ball scoop' was used to cut out the eye from the rose end of each tuber, taking care to avoid damage to the sprouts. The resultant potato balls were left to suberise for at least 72 hours. The potato balls were then immersed in 1 % sodium hyper-chlorite for 5 minutes and rinsed twice in distilled water before being allowed to air dry.

The potato balls were planted in pots (plastic 'vending' cups, height: 8 cm, bottom diameter: 4 cm). Each pot had a 5 mm diameter hole drilled into the centre of the base and a layer of fine mesh (gauge: 0.5 mm) placed over the hole. This provided drainage for leachates but prevented potting medium from escaping. Thirty pots were partially filled with ca. 200g of twice autoclaved silver sand. By using a sterile stirring rod, a 4 cm deep, 2.5 cm wide hole was dibbed into the centre of 20 sand filled pots to allow a potato ball to be planted. The 10 remaining pots were left unplanted and served as controls for the potting medium. Following planting the pots were transferred to a controlled environment cabinet (Conviron Model S10H, Controlled Environments Ltd, Winnipeg, Manitoba, Canada, R3H OW9), which was programmed with a 15 hour photoperiod (06:00-21:00), a daytime temperature of 18°C, a

night time temperature of 10°C, and a relative humidity of 50% to represent springtime conditions in the UK. Pots were arranged in 10 randomised blocks with the treatments shown in Table 5.1. Treatments of *G. rostochiensis* were implemented (as described below) three weeks after planting when the potato plants had fully emerged. The pots were watered every 72 hours prior to emergence and every 48 hours following emergence by using a syringe to administer 20 ml of sterile distilled water to each pot.

Table 5.1 Experimental treatments used to produce leachates from plants infested and uninfested with *G. rostochiensis* in controlled environment experiment 1. Pots not containing a potato plant served as a control for the potting medium

Treatment number	Potato plant	Level of *G. rostochiensis* infestation (number of juveniles applied to each pot)
1	-	0
2	+	0
3	+	9990

Unlike previous experiments of this project, juveniles as opposed to cysts of *G. rostochiensis* were used in nematode treatments. It was hoped that a pre-determined number of hatched, viable juveniles would produce a more uniform invasion of potato roots in each treatment group. The level of *G. rostochiensis* infestation shown in Table 5.1 was based upon soil densities of 100 eggs g^{-1} soil and the assumption of a 50 % hatch rate (Perry *et al.*, 1992). Juveniles of *G. rostochiensis* were hatched from cysts using potato root leachates (Clarke & Hennessy, 1984; 1987) as outlined below.

Approximately 10 kg of soil infested with *G. rostochiensis* was collected from 'Four-Gates Field', Harper Adams University College (Ordnance Survey Grid Reference: SJ 707195) and then dried at 25°C for 4-5 days. *Globodera rostochiensis* densities were determined from three 200 g sub-samples of the soil bulk using the methods described in Chapter 2 (pages 60-61). By using equation 2 (see Chapter 3, page 66) it was calculated that 2297 cysts would be required. However, to ensure an adequate supply of juveniles, ca.7000 cysts were extracted from the soil. The cysts were divided into groups of 100, placed into watch glasses containing 2 ml of distilled water, covered with a cover glass and placed in an incubator at 15 °C for 3 days. Following incubation, the watch glasses were removed from the incubator and examined for the presence of floating cysts. Watch glasses containing floating cysts were mixed with a Pasteur pipette via suction and expulsion until all the cysts were fully immersed in water. The watch glasses were then covered and returned to the incubator for a further 4 days. Following incubation, a Pasteur pipette was used to remove the water from each of the watch glasses. The cysts were then removed from the watch glasses and divided equally into 10 sterile Petri dishes each containing 14 ml of potato root leachate. Potato root leachate (PRL) was collected from 3-week-old potato plants (cv. Désirée) using the methods of Fenwick (1949) and Dr S.K. Ibrahim, Harper Adams University College (Pers. Comm.). The Petri dishes containing the cysts and PRL were incubated for 5 days at 15 °C and examined on a daily basis with a binocular microscope (magnification = x 30) to monitor their progress. Following incubation, sufficient juveniles had hatched for the requirements of the experiment. The contents of the 10 Petri dishes were passed through two sieves into a 250 ml glass beaker (Figure 5.2). The upper sieve (gauge: ca. 300 µm) was used to collect the cysts whilst the lower sieve (gauge: 10 µm) collected the juveniles. The juveniles in the lower sieve were washed 3 times with distilled water. The lower sieve was then inverted over a glass funnel on a 250 ml volumetric flask and the contents were flushed into the flask via a stream of sterile distilled water. Once all the

juveniles had been transferred, the flask was made up to 250 ml with sterile distilled water and was moved to a magnetic stirrer, which was set on a low stir. Three 2 ml samples were removed from the juvenile stock suspension and transferred to DeGrisse counting slides using a 5 ml pipette. Each slide was examined under a binocular microscope (x 30) to obtain an estimate of viable juveniles ml^{-1} water.

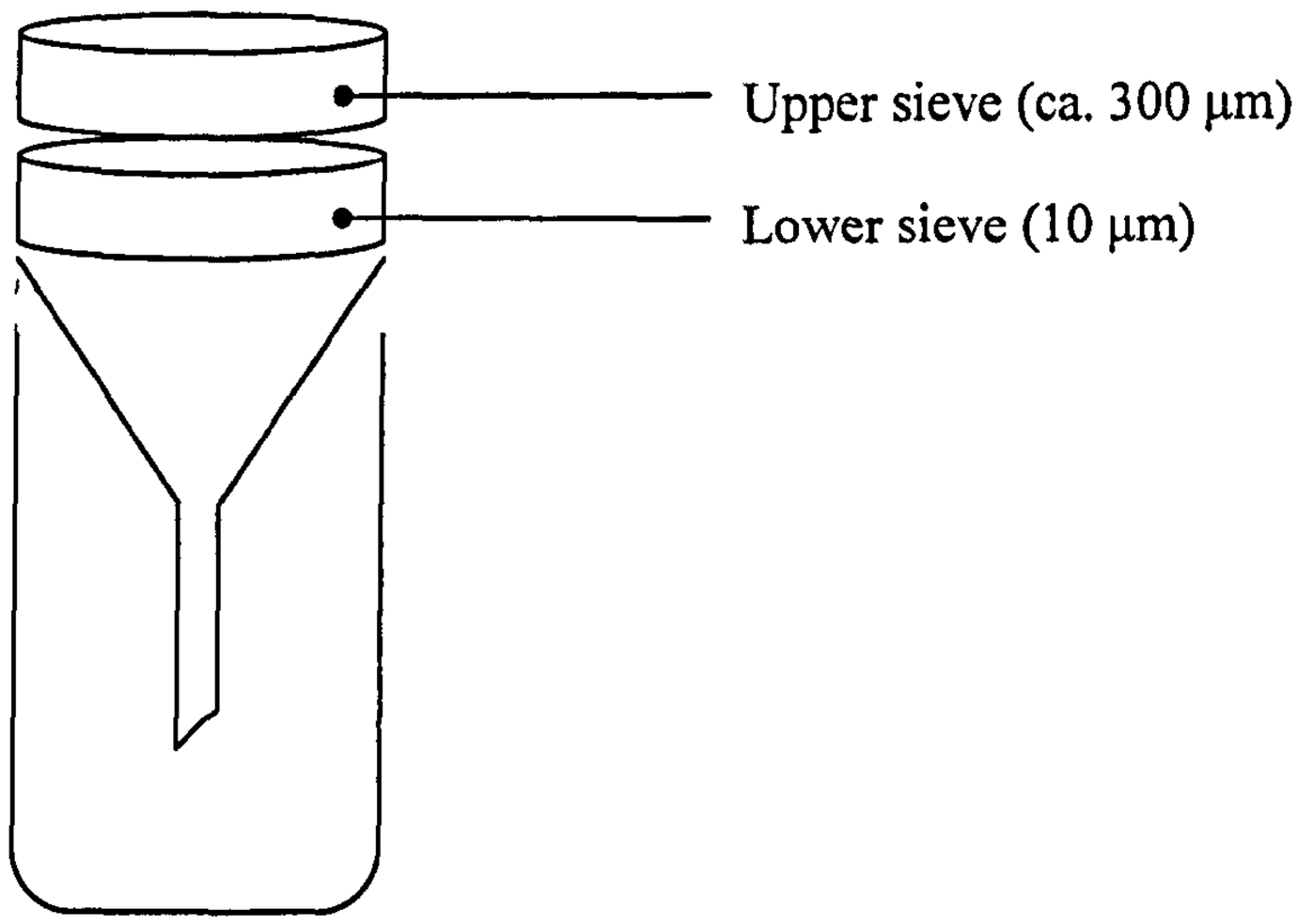


Figure 5.2 Apparatus used to extract and wash *G. rostochiensis* juveniles following hatching in potato root leachate

Since the quantity of *G. rostochiensis* juveniles within the stock suspension had been pre-determined, the volume of juvenile suspension required for infestation (Table 5.1) could be calculated. As previously mentioned, potato plants within the controlled environment cabinet had been grown for three weeks before nematode infestation treatments were undertaken. Prior to infestation, a stirring rod was used to make 4 holes, 3 cm deep in the potting medium around each of the potato plants (Figure 5.3). Soil was infested, by using a pipette to gently

apply equal volumes of the calculated quantity of juvenile suspension to each hole. To ensure that each plant received equal volumes of sterile distilled water, an additional quantity of sterile distilled water was added to make the total volume of distilled water applied up to 20 ml in each pot. After each pot had been treated, the holes were closed with a stirring rod and the pot was returned to its designated position in the controlled environment cabinet. During the intervals between applying treatments, the juvenile suspension was maintained on a magnetic stirrer with a low stir. In treatments where no nematode infestation was required, 20 ml of sterile distilled water was applied.

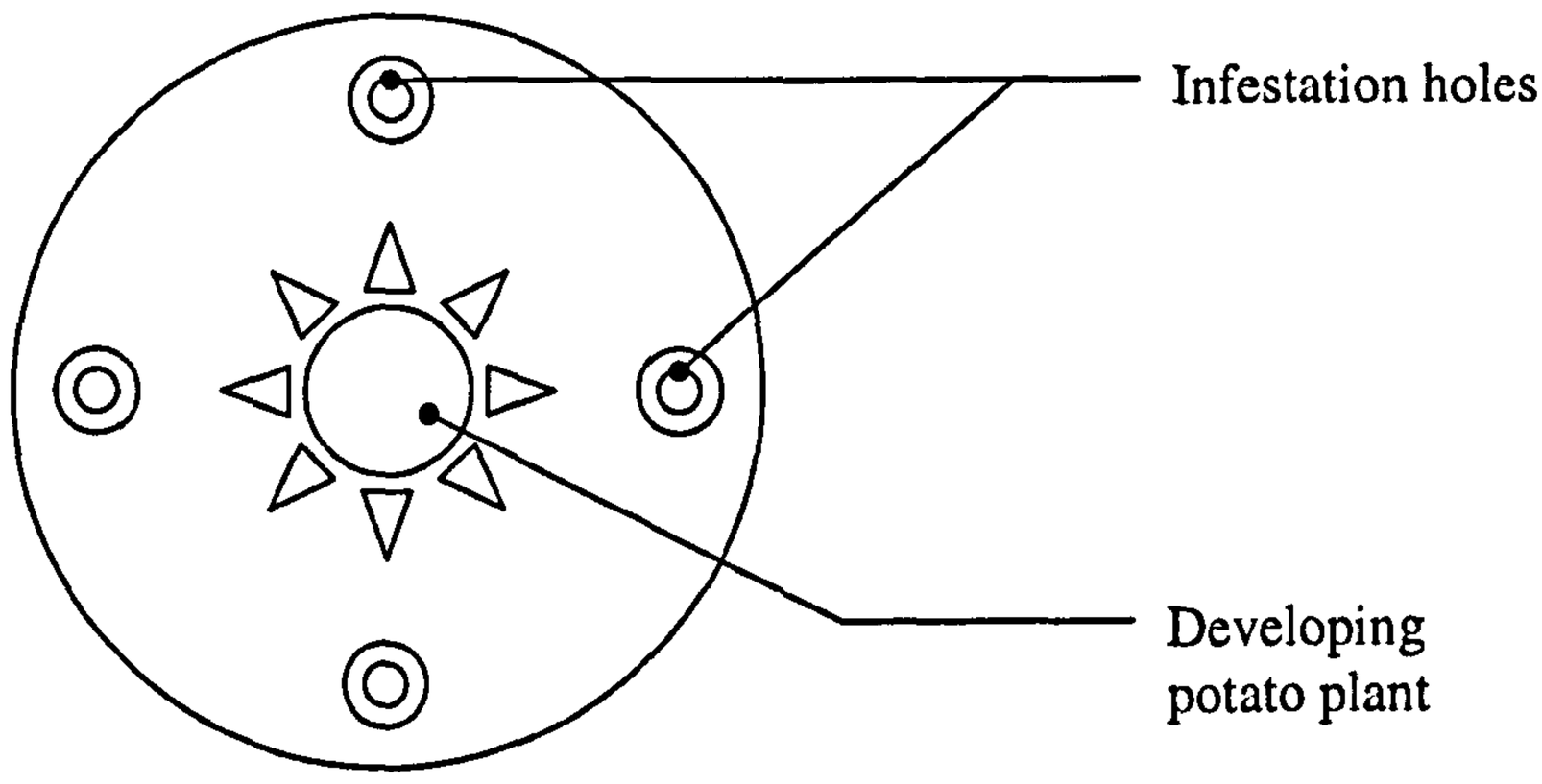


Figure 5.3 An aerial view of an experimental pot indicating the location of holes used to apply *G. rostochiensis* juvenile suspension

Following nematode infestation, the 48-hourly watering regime was resumed and the pots were given a weekly 20 ml dose of NPK fertiliser (J. Arthur Bowers liquid fertilizer, Firth road, Lincoln, LN6 YAH) – 0.5 ml fertiliser/l distilled water.

Root leachates were collected 4, 6, 8, 12 and 18 days after the nematode infestation treatments were administered. Prior to leachate collection the pots were left without water for 48 hours. Leachates were collected with the aid of specialised racks, which comprised an upper shelf for holding 10 pots, a middle shelf holding short-necked funnels and a lower shelf with 25 ml sample tubes for collecting the leachates (Plate 5.1). Each of the funnels was lined with Whatman ® No. 1 filter paper (gauge: 11 μm). Pots were removed from the controlled environment cabinet in blocks and placed on an assembled rack with correspondingly labelled sampled tubes beneath them. A sterile syringe was used to administer sufficient sterile distilled water to saturate each of the pots (ca. 40 ml). Following a 5 minutes interval, a further 25 ml of sterile distilled water was applied to each pot. When each sample tube was full it was removed from the rack and a new tube was placed underneath to collect any excess leachates. When all the leachates had been collected the pots were returned to the controlled environment cabinet and the sample tubes were taken back to the laboratory for processing. Between leachate collection dates, the soiled glassware was soaked in 3 % Decon 90 ® for an hour, rinsed and then autoclaved.

Each sample tube was shaken by hand for 10 seconds before being filtered using a syringe attached to a 0.2 μm Millipore® filter to remove any bacteria (Agrios, 1988). Twenty millilitre samples were removed from each filtered sample and transferred to 25ml disposable Sterilin® tubes and maintained at 4°C.

When the last root leachate collection had been made, the plants were removed from their pots, washed, placed in labelled polythene bags and taken back to the laboratory. The root system of each plant was cut away, weighed and preserved for the determination of *G. rostochiensis* juvenile densities at a later date according to the procedures outlined in Chapter 2, (page 62).

Plate 5.1 Rack assemblage used for collecting leachates from potato plants (cv. Désirée) grown in controlled environment experiments

5.2.1.2 *In-vitro* experiments investigating the effect of root leachates from potato plants infested and uninfested with *G. rostochiensis* on the radial growth of *R. solani*

A series of 5 *in-vitro* experiments were conducted to compare the growth rate of *R. solani* isolates on media either amended with root leachates from *G. rostochiensis* infested or amended with root leachates from uninfested potato plants. Since the root leachates were collected at different times after *G. rostochiensis* infestation treatments, separate experiments were conducted for each leachate collection date. Each experiment included a treatment where *R. solani* isolates were grown on media amended with leachates from pots with no potato plants but treated with NPK fertiliser. This treatment was used to provide a comparison between the potting medium with and without potato plants.

Five *R. solani* (AG 3) isolates of different geographical origins were obtained from the Harper Adams University College culture collection (Table 5.2). Prior to each experiment, the isolates were sub-cultured onto Petri dishes containing potato dextrose agar amended with streptomycin sulphate and incubated at 15°C for 7 days.

Four 1 litre Schott ® bottles containing 800 ml of distilled water and 10 g of Agar No. 2 (Lab M) (water agar) were autoclaved for 20 minutes at 121°C. On cooling (ca. 45°C), each bottle of media was amended with 200 ml of either leachate from pots containing potato plants infested with *G. rostochiensis*, leachate from pots containing potato plants uninfested with *G. rostochiensis*, leachate from pots containing no potato plant or sterile distilled water. Each 200 ml of leachate was made from a composite of 10 x 20 ml samples that were collected from individual replicate pots. The amended media was poured into 50 sterile Petri dishes (plates), at an approximate rate of 25 ml/plate. When set, the underside of each agar plate was marked

with a central cross (see Figure 5.4). Ten replicate plates of each medium were inoculated with each of the 7-day-old *R. solani* isolates by placing a 6 mm *R. solani* plug in the centre of each plate. The plates were then sealed with Parafilm ®, arranged into 10 randomised blocks (stacks) and incubated at 15 °C. The diameter of each growing colony was measured along both the vertical and horizontal marked lines of each plate on a daily basis (Figure 5.4). A mean of the two measurements was calculated in order to obtain a single value of radial growth. The experiment was run until a colony was found to have reached the edge of a plate. This procedure was repeated for each day that leachates were collected (collection dates).

Table 5.2 Details of *R. solani* (AG 3) isolates used in *in-vitro* experiments investigating the effect of root leachates from potato plants (cv. Désirée) infested and uninfested with *G. rostochiensis* on the radial growth of *R. solani*. All isolates were originally isolated from black scurf on potato tubers

Isolate code	Origin
I3	Shropshire, UK
X44	Yorkshire, UK
X50	Scotland, UK
X56	Cornwall, UK
X62	Suffolk, UK

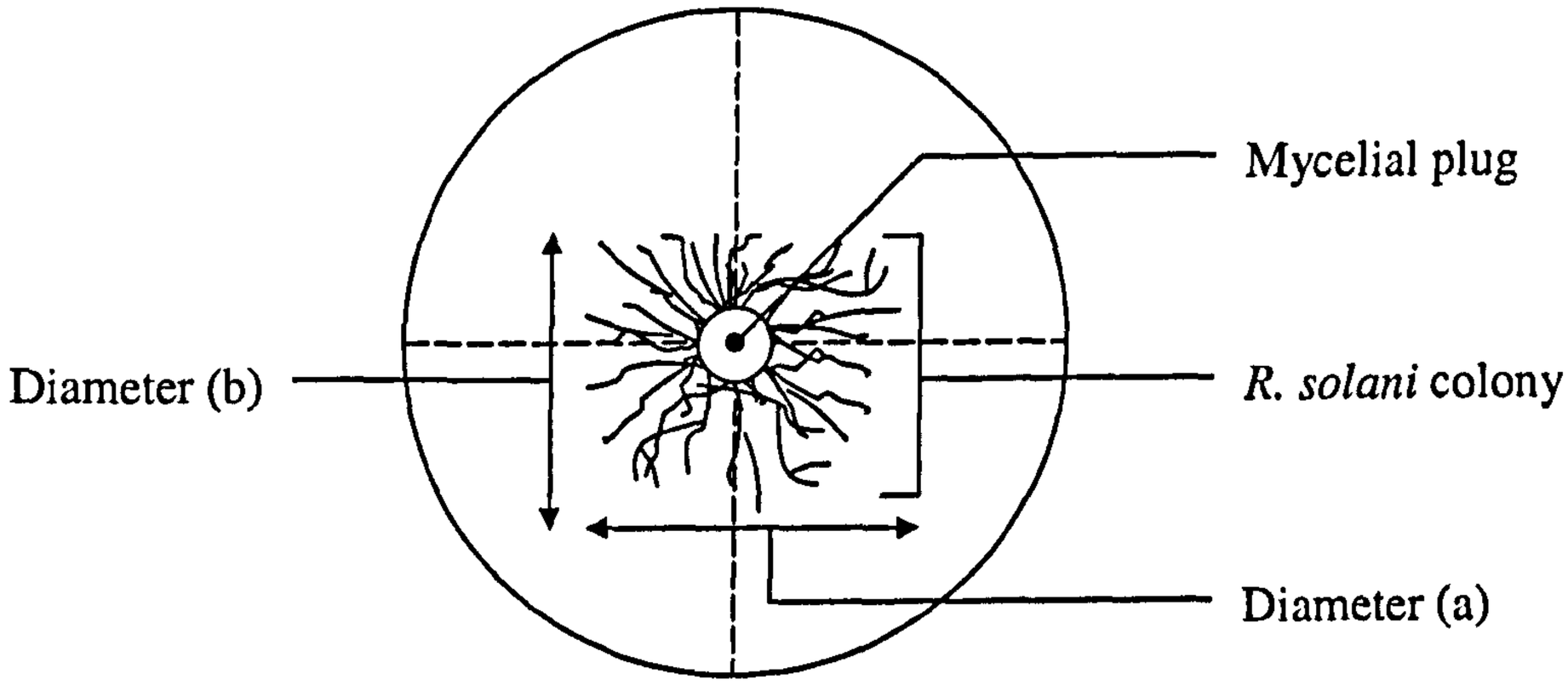


Figure 5.4 An illustration of how the radial growth of *R. solani* (AG 3) was measured during *in-vitro* experiments investigating the effect of root leachates from potato plants (cv. Désirée) either infested or uninfested with *G. rostochiensis* on the radial growth of *R. solani*. A mean of diameter's (a) and (b) was calculated to obtain a single measurement of radial growth

5.2.2 Experiment 2

5.2.2.1 Root leachate collection and *R. solani* radial growth experiments

Following the procedure outlined in experiment 1, a second controlled environment was set up with the treatments shown in Table 5.3, replicated in 15 blocks. The potato seed stock (cv. Désirée), controlled environment cabinet settings and *G. rostochiensis* infested soil was identical to that used in the initial experiment. Pots were watered every 72 hours prior to emergence and every 48 hours following emergence by using a syringe to administer 20 ml of sterile distilled water to each pot. Unlike the first experiment, plants were not given any nutrient supplements. Leachates were collected as previously described but were filtered by Whatman ® No. 5 filter paper (gauge: 2.5 μm) in the rack assemblage and were not subsequently filtered by 0.2 μm Millipore® filters. In addition to taking a 20 ml sample, two 2 ml samples were placed in Eppendorf tubes and stored at -80°C for subsequent carbohydrate and nitrogen analyses.

Table 5.3 Experimental treatments used to produce leachates of plants infested and uninfested with *G. rostochiensis* in controlled environment experiment 2. Pots not containing a potato plant served as a control for the potting medium

Treatment number	Potato plant	Level of *G. rostochiensis* infestation (number of juveniles applied to each pot)
1	-	0
2	+	0
3	+	15000

Leachates were collected 4, 8 and 12 days after infestation with *G. rostochiensis* juveniles. At each leachate collection date, an *in-vitro* radial growth experiment was conducted using the procedure and *R. solani* isolates detailed in experiment 1. However, several modifications were introduced with the aim of optimising the experiment. Firstly the quantity of leachate used to amend agar was increased to 300 ml (i.e. 15 x 20 ml replicates). The agar was accurately dispensed at 10 ml/plate by using a sterile 100 ml syringe attached to a 30 cm length of plastic tubing. In between treatments the syringe and tubing was soaked in 1 % sodium hyperchlorite for 3 minutes and rinsed 3 times with sterile distilled water. The plates were incubated at 10˚C and measured every 48 hours.

5.2.2.2 Determination of sugar content within potato root leachates

The sugar content of the leachate samples was determined by adapting the enzyme-coupled microplate method of Spackman & Cobb (2001). Prior to analysis, leachate samples were retrieved from storage at –80˚C and allowed to thaw at room temperature. When thawed, 200 µl of each leachate sample was added to 3 vertically adjacent wells (strips) of a Falcon ® flat-bottomed, 96-well microtitre plate (Farenheit, Northampton, UK) in triplicate (Figure 5.5). On each plate, a block of 15 wells (5 x 3 well strips) was reserved for standard sugar solutions of glucose, fructose and sucrose at concentrations of 0, 25, 50, 75 and 100 µg ml^{-1}, which were assayed at 10 µl per well. The outer wells of the plate were not used to avoid any edge effects. When the plate was loaded with both samples and standards, the absorbance of each well was determined using a Benchmark ® microplate reader (BioRad, Hemel Hempstead, UK), which was programmed to shake the plate for 20 seconds before reading at 340 nm.

A 30-µl dose of glucose reagent (Bayer, Newbury, UK) was added to the first well of each strip (Figure 5.5) in order to produce an enzymic oxidation of the sample glucose to 6-phosphogluconic acid coupled with a reduction of NAD to NADH (Spackman & Cobb, 2001). Subsequently, a 30-µl dose of glucose reagent amended with 0.2 units of phosphoglucose isomerase, type IV from rabbit muscle (Sigma, Poole, UK) was added to the second and third wells of each strip (Figure 5.5). Spackman & Cobb (2001), state that phosphoglucose isomerase converts fructose to 6-phosphogluconic acid. The third well of each strip (Figure 5.5) was additionally treated with 10 µl of invertase grade VII from bakers yeast (Sigma, Poole, UK) in 0.1 mol sodium acetate (pH 5), which converts sucrose to 6-phosphogluconic acid (Spackman & Cobb, 2001). The plate was incubated at room temperature for 40 minutes before the absorbance at 340 nm was determined. A repeat reading was taken 60 minutes after the enzymes were applied. The concentration of each sugar was determined by an increase in the concentration of 6-phosphogluconic acid via enzyme mediated activity. Subsequently, this biochemical change determined the absorbance of each well. Increases in absorbance, were determined by subtracting the initial absorbance from the final absorbance. The sugar concentration of each well was quantified by comparing the increase in sample absorbance with the absorbance of standard sugar solutions. Using the absorbance results of the standard sugar solutions a standard curve was produced for each sugar. The quantity of each sugar per ml of leachate was calculated using equation (5) where y = the increase in sample absorbance, m = the gradient of the standard curve, c = the intercept of the standard curve and x = the quantity of sugar in µg ml^{-1} sample.

$$x = (y/m) - c \qquad \text{equation (5)}$$

The increase in sample absorbance was calculated using the difference between readings taken at 0 and 40 minutes for glucose and fructose and the difference between readings taken at 0 and 60 minutes for sucrose. Sucrose was given a longer interval between readings to enable the optimum pH for invertase to metabolise hexoses (Spackman & Cobb, 2001).

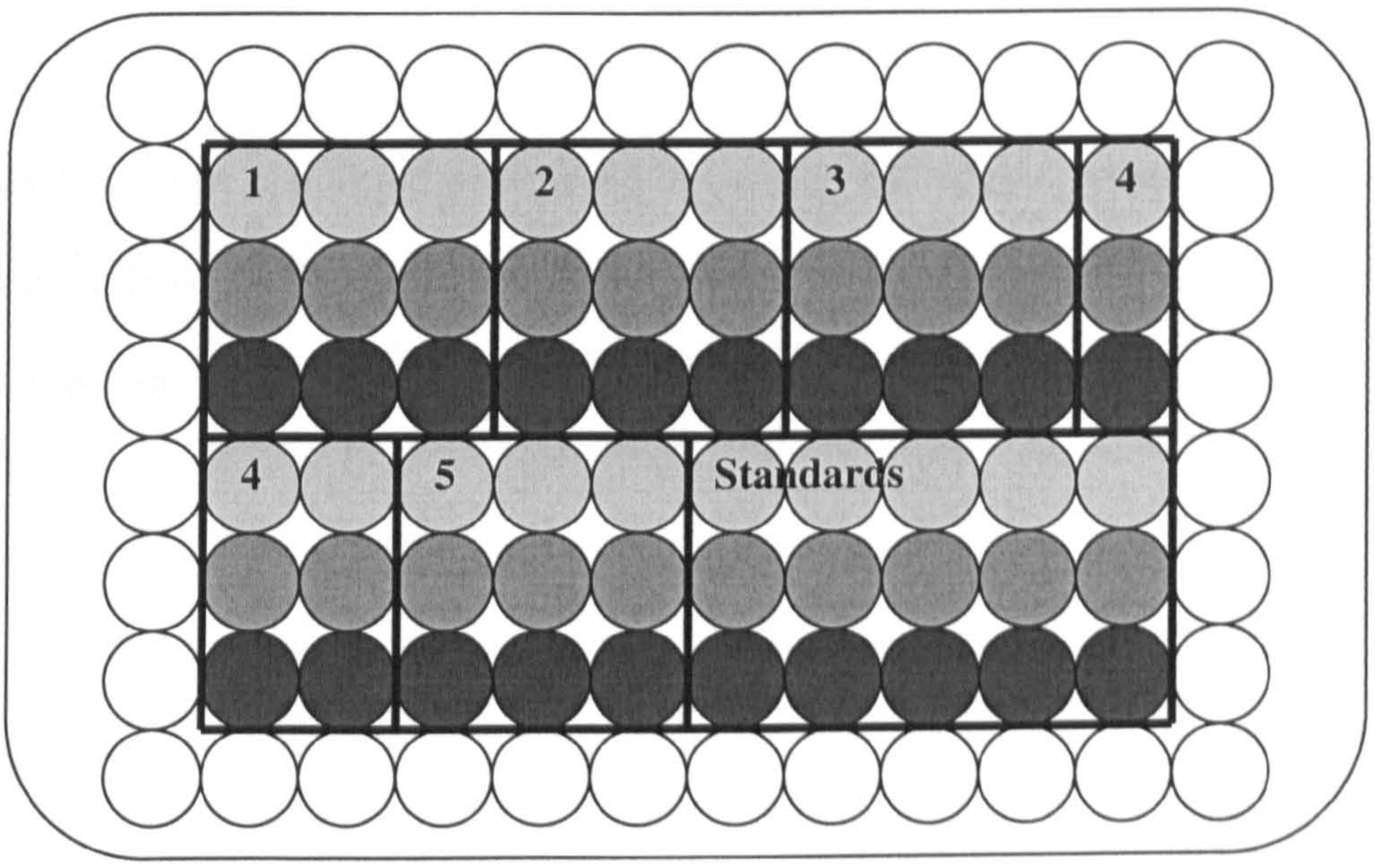


Figure 5.5 Diagram of an enzyme coupled-microplate assay showing the position of wells containing glucose reagent (●), glucose reagent amended with phosphoglucose isomerase(●), glucose reagent amended with phosphoglucose isomerase + invertase(●). The samples were loaded in triplicate (blocks 1-5) and standard sugar solution concentrations (standards) were included in each plate. The outer wells (○) were not used

5.2.2.3 Determination of nitrogen content

The nitrogen content of leachates was determined using Leco® FP-528 combustion based nitrogen/protein apparatus (Leco Corporation, 3000 Lakeview Avenue, St Joseph, MI 49085, USA) as per manufacturer's instructions.

5.2.3 Statistical analysis

All statistical analyses were conducted with the aid of Genstat – 5th edition, release 4.2 (2000), Lawes Agricultural Trust ©. Frequency histograms were used to examine the distribution within variates and data not showing a typical normal/Gaussian distribution was transformed.

5.3 Results

5.3.1 Experiment 1

5.3.1.1 *In-vitro* experiments investigating the effect of root leachates from potato plants infested and uninfested with *G. rostochiensis* on the radial growth of *R. solani*

Tables 5.4-5.6 show the mean radial growth of *R. solani* isolates on agar amended with root leachates from potato plants either infested or uninfested with *G. rostochiensis*. The mean radial growth of *R. solani* isolates, 72-96 hours after inoculation was significantly higher ($P<0.001$) on agar amended with root leachates from *G. rostochiensis* infested plants compared to agar amended with root leachates from uninfested plants, when root leachates were collected 4 days after the introduction of *G. rostochiensis* juveniles. However, the highest recording of *R. solani* radial growth was found 24-96 hours after inoculation on agar amended with leachates collected from pots containing sand and NPK fertiliser. Subsequent radial growth experiments using potato root leachates collected 6, 8 and 12 days after the introduction of *G. rostochiensis* juveniles produced similar results to the 4-day collection. The final radial growth experiment using root leachates collected from plants 18 days after the introduction of *G. rostochiensis* revealed fewer significant differences between experimental treatments. In particular, the growth of *R. solani* isolates on agar amended with root leachates from plants infested with *G. rostochiensis* compared to agar amended with root leachates from uninfested plants was not significantly different 24, 72 and 96 hours after the isolates were placed onto the agar.

Table 5.4 The mean radial growth of *R. solani* isolates grown on unamended water agar, water agar amended with leachates from pots containing sand, water agar amended with leachates from potato plants (cv. Désirée) uninfested or infested with *G. rostochiensis*. Leachates were collected (a) 4 and (b) 6 days after *G. rostochiensis* treatments were administered in experiment 1

(a)

Treatment	Mean radial growth of *R. solani* isolates			
	24h*	48h*	72h*	96h*
Water agar – unamended	-	-	-	-
Water agar – amended with leachates from pots containing silver sand only	9.04b	24.96b	42.29c	57.84c
Water agar – amended with leachates from potato plants (cv. Désirée)	7.33a	19.81a	33.36a	46.89a
Water agar – amended with leachates from potato plants (cv. Désirée) infested with *G. rostochiensis* juveniles	7.45a	20.90a	36.04b	51.13b
d.f.	138	138	138	138
S.E.M.	0.256	0.44	0.59	0.72
L.S.D. (*P*=0.001)	0.620	1.158	1.382	2.291
%cv	19.7	13.4	9.4	11.1

* Numbers followed by the same letter are not significantly different according to LSD multiple range test

(b)

Treatment	Mean radial growth of *R. solani* isolates			
	24h*	48h*	72h*	96h*
Water agar – unamended	8.31a	18.37a	30.41a	43.66a
Water agar – amended with leachates from pots containing silver sand only	9.53b	21.45b	35.44c	52.22d
Water agar – amended with leachates from potato plants (cv. Désirée)	8.27a	18.69a	30.24a	45.84b
Water agar – amended with leachates from potato plants (cv. Désirée) infested with *G. rostochiensis* juveniles	8.51a	20.07a	33.06b	49.16c
d.f.	187	187	187	187
S.E.M.	0.299	0.44	0.51	0.60
L.S.D. (*P*=0.001)	0.896	1.317	1.463	1.491
%cv	26.2	17.0	11.5	7.9

* Numbers followed by the same letter are not significantly different according to LSD multiple range test

Table 5.5 The mean radial growth of *R. solani* isolates grown on unamended water agar, water agar amended with leachate from pots containing sand, water agar amended with leachates from potato plants (cv. Désirée) uninfested or infested with *G. rostochiensis*. Leachates were collected (a) 8 and (b) 12 days after *G. rostochiensis* treatments were administered in experiment 1

(a)

Treatment	Mean radial growth of *R. solani* isolates			
	24h	48h*	72h*	96h*
Water agar – unamended	9.27a	20.30a	31.37a	43.74a
Water agar – amended with leachates from pots containing silver sand only	11.49b	25.86c	41.06d	57.52d
Water agar – amended with leachates from potato plants (cv. Désirée)	9.85a	21.44a	33.58b	47.62b
Water agar – amended with leachates from potato plants (cv. Désirée) infested with *G. rostochiensis* juveniles	11.07b	23.37b	36.37c	51.70c
d.f.	187	187	187	187
S.E.M.	0.247	0.37	0.46	0.57
L.S.D. (P=0.001)	0.787	1.188	1.437	1.680
%cv	19.1	13.2	10.2	8.5

* Numbers followed by the same letter are not significantly different according to LSD multiple range test

(b)

Treatment	Mean radial growth of *R. solani* isolates			
	24h*	48h*	72h*	96h*
Water agar – unamended	6.48a	17.04a	27.43a	40.18a
Water agar – amended with leachates from pots containing silver sand only	7.73b	21.25c	35.54c	51.94c
Water agar – amended with leachates from potato plants (cv. Désirée)	6.59a	17.00a	28.00a	41.61a
Water agar – amended with leachates from potato plants (cv. Désirée) infested with *G. rostochiensis* juveniles	7.75b	19.28b	31.73b	46.66b
d.f.	187	187	187	187
S.E.M.	0.199	0.36	0.49	0.61
L.S.D. (P=0.001)	0.705	1.156	1.410	1.676
%cv	25	15.7	11.6	9.4

* Numbers followed by the same letter are not significantly different according to LSD multiple range test

Table 5.6 The mean radial growth of *R. solani* isolates grown on unamended water agar, water agar amended with leachate from pots containing sand only, water agar amended with leachates from potato plants (cv. Désirée) uninfested or infested with *G. rostochiensis*. Leachates were collected 18 days after *G. rostochiensis* treatments were administered in experiment 1

Treatment	Mean radial growth of *R. solani* isolates			
	24h*	48h*	72h*	96h*
Water agar – unamended	10.10a	21.71a	35.30a	48.77a
Water agar – amended with leachates from pots containing silver sand only	11.16b	23.72c	38.83b	54.48c
Water agar – amended with leachates from potato plants (cv. Désirée)	9.84a	20.85a	34.49a	50.09ab
Water agar – amended with leachates from potato plants (cv. Désirée) infested with *G. rostochiensis* juveniles	10.55ab	22.21b	35.30a	51.15b
d.f.	187	187	187	187
S.E.M.	0.191	0.27	0.31	0.40
L.S.D. (P=0.001)	0.733	1.026	1.194	1.572
%cv	17.8	11.7	8.4	7.8

* Numbers followed by the same letter are not significantly different according to LSD multiple range test

5.3.1.2 Invasion of potato roots by *G. rostochiensis* juveniles

The invasion of potato roots by *G. rostochiensis* juveniles in experiment 1 is shown in Table 5.7 As expected, no juveniles were found in plants uninfested with *G. rostochiensis*. Although the invasion of roots was generally lower than expected, the treatment groups were well defined in terms of the number of juveniles that had successfully invaded the potato roots. Figure 5.6 shows the distribution of juvenile stages found.

Table 5.7 The invasion of potato roots (cv. Désirée) by *G. rostochiensis* juveniles in controlled environment experiment 1

Treatment	Mean *G. rostochiensis* juveniles g^{-1} root	S.E.M.	%cv
Uninfested potato plants	0	0	-
Potato plants infested with *G. rostochiensis* juveniles	572.5	57.0	31.5

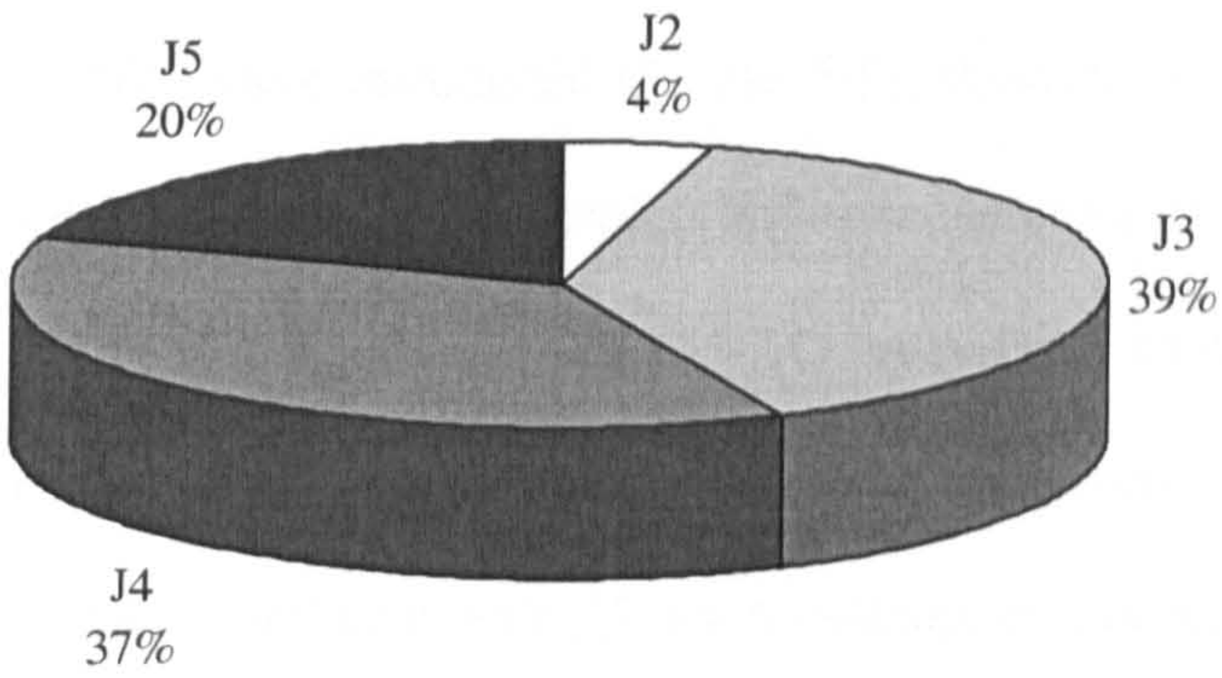


Figure 5.6 The mean distribution of *G. rostochiensis* juvenile stages (J2-J5) recovered from potato (cv. Désirée) roots, 18 days after their introduction to 2-week-old plants in experiment 1

5.3.2 Experiment 2

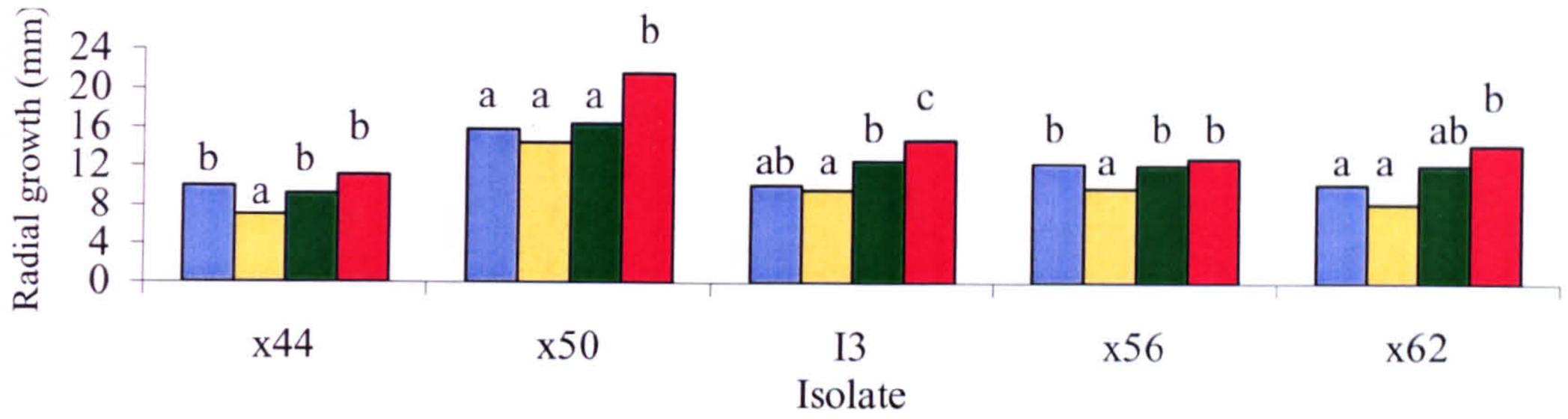
5.3.2.1 *In-vitro* experiments investigating the effect of root leachates from potato plants infested and uninfested with *G. rostochiensis* on the radial growth of *R. solani*

Figures 5.7-5.9 present the radial growth of *R. solani* isolates on water agar either unamended, amended with leachate from pots containing silver sand only, amended with root leachates from plants uninfested with *G. rostochiensis* or amended with root leachates from plants infested with *G. rostochiensis*. The first experiment (Figure 5.7), conducted with leachates collected 4 days after *G. rostochiensis* juveniles were introduced, revealed that *R. solani* isolates X50 and I3 had a significantly greater growth on agar amended with root leachates from potatoes infested with *G. rostochiensis* compared to all other treatments. In addition, *R. solani* isolates X44 and X62 had a significantly greater growth on agar amended with root leachates from plants infested with *G. rostochiensis* compared to agar amended with root leachates from uninfested plants.

The second radial growth experiment conducted with leachates collected 8 days after *G. rostochiensis* juveniles were introduced (Figure 5.8), showed that the radial growth of all *R. solani* isolates was higher on agar amended with root leachates from plants infested with *G. rostochiensis* than on agar amended with root leachates from uninfested plants. Furthermore, *R. solani* isolates I3 and x56 had a significantly greater growth on agar amended with root leachates from potatoes infested with *G. rostochiensis* compared to all other experimental treatments. The final radial growth experiment conducted with leachates collected 12 days after *G. rostochiensis* juveniles were introduced (Figure 5.9), showed that the radial growth of all *R. solani* isolates was greater on agar amended with root leachates from potato plants

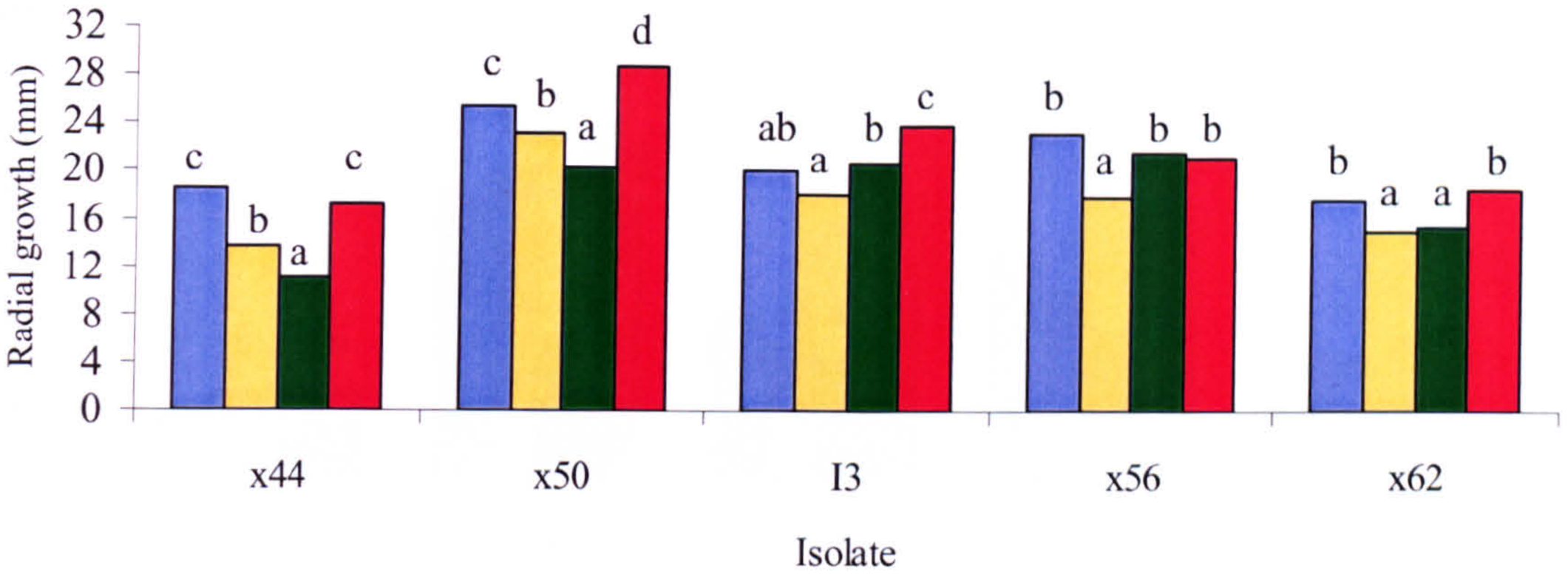
infested with *G. rostochiensis* than that of all other experimental treatments. This effect was more pronounced at 96 and 144 hours after inoculation with *R. solani* isolates.

(a) 48h



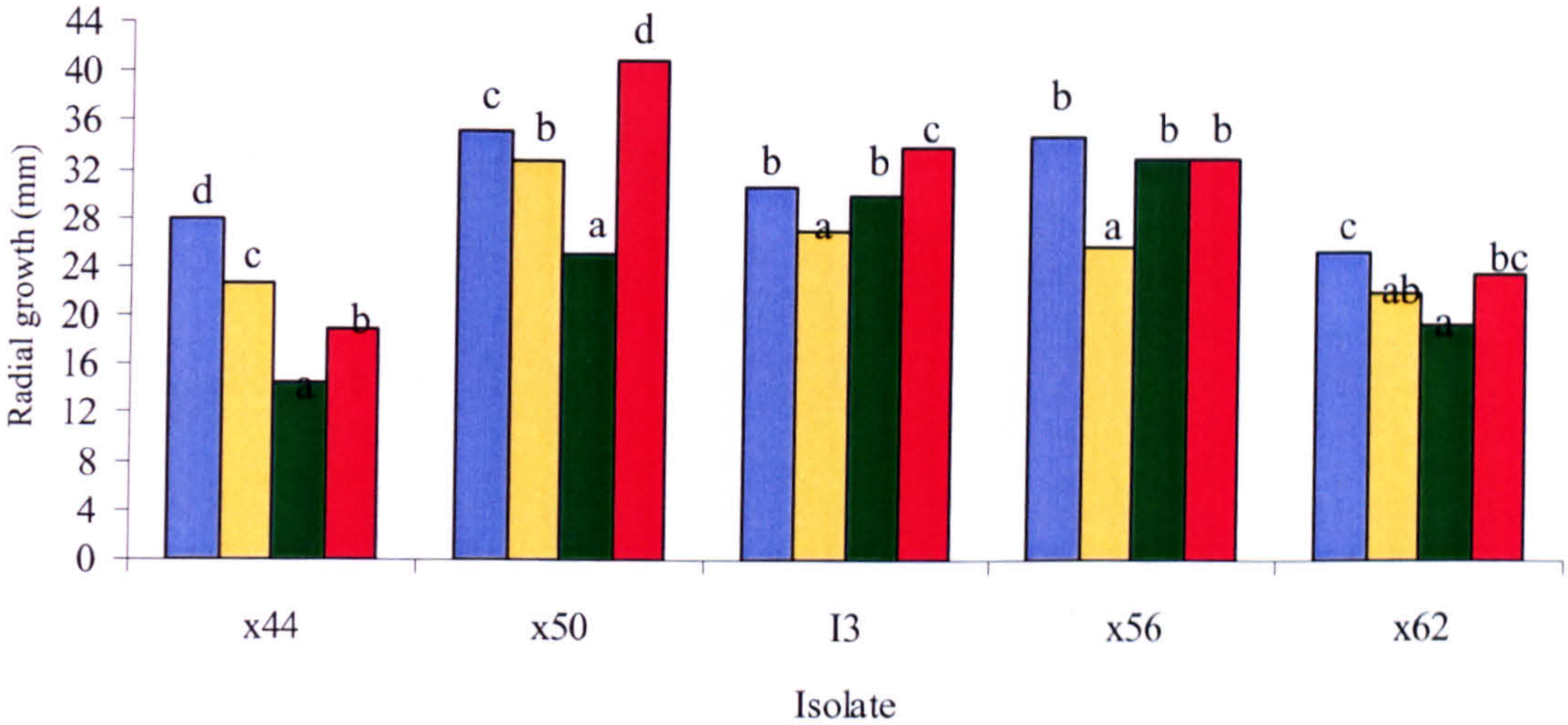
d.f. = 171 S.E.M. = 0.29 L.S.D. (P=0.001) = 2.092 %cv = 19.3

(b) 96h



d.f. = 171 S.E.M. = 0.34 L.S.D. (P=0.001) = 2.338 %cv = 13.5

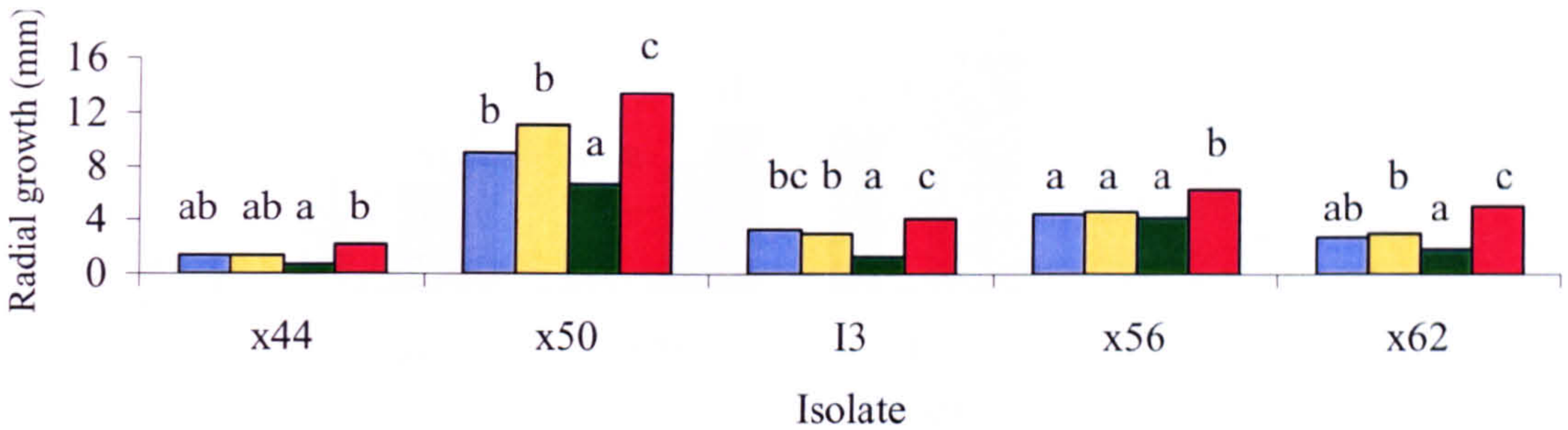
(c) 144h



d.f. = 171 S.E.M. = 0.52 L.S.D. (P=0.001) = 3.193 %cv = 13.0

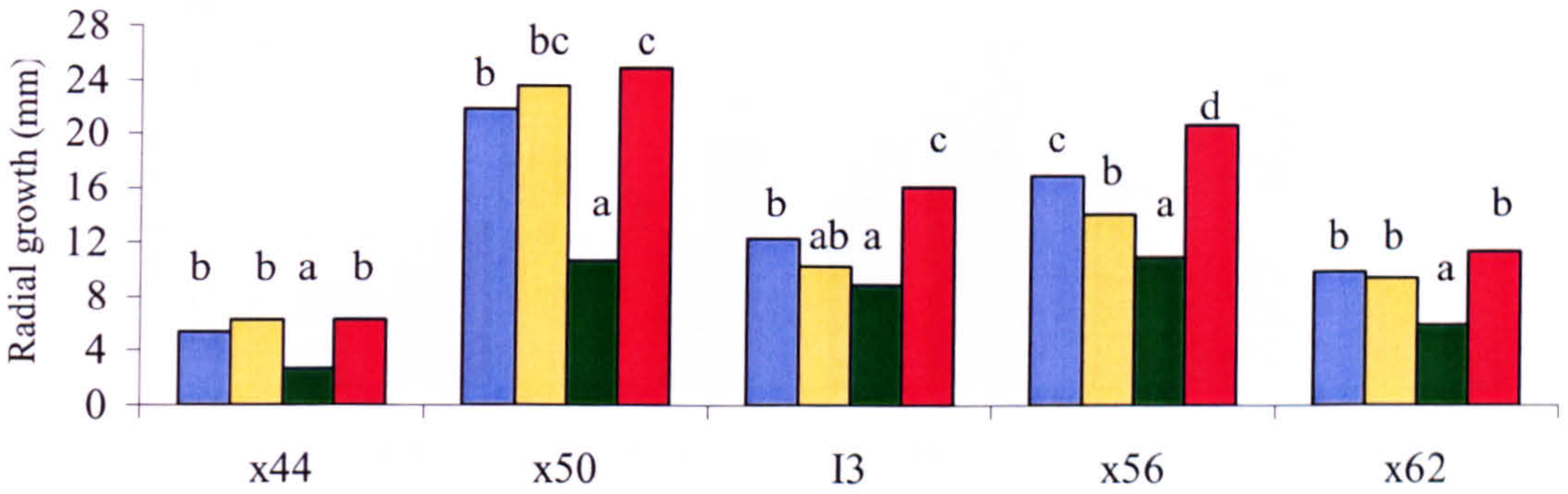
Figure 5.7 The radial growth of *R. solani* isolates grown on unamended water agar (■), water agar amended with leachate from pots containing silver sand only (■), water agar amended with leachates from potato plants (cv. Désirée) uninfested (■) or infested with *G. rostochiensis* (■) at (a) 48, (b) 96 and (c) 144 hours after inoculation with *R. solani* isolates. Leachates were collected 4 days after *G. rostochiensis* juveniles were introduced. Bars with the same letter are not significantly different according to L.S.D. statistics.

(a) 48h



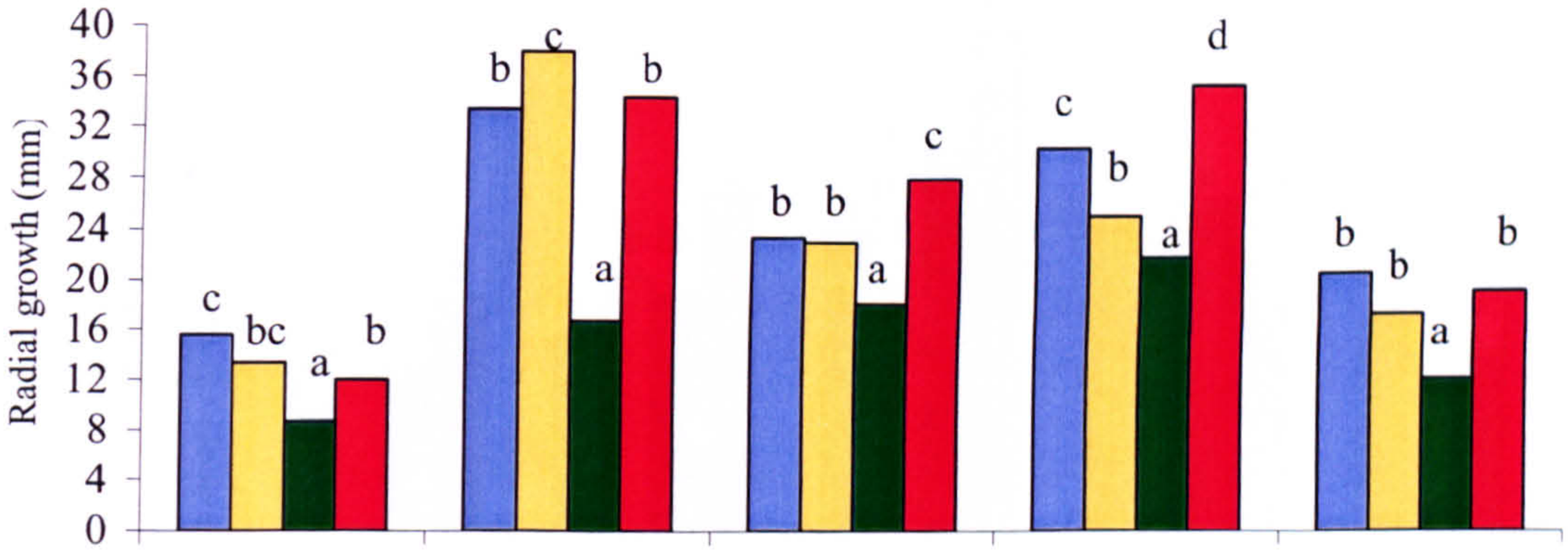
d.f. = 171 S.E.M. =0.246 L.S.D. (P=0.001) = 0.9956 %cv = 25

(b) 96h



d.f. = 171 S.E.M. = 0.48 L.S.D. (P=0.001) = 2.234 %cv = 20.4

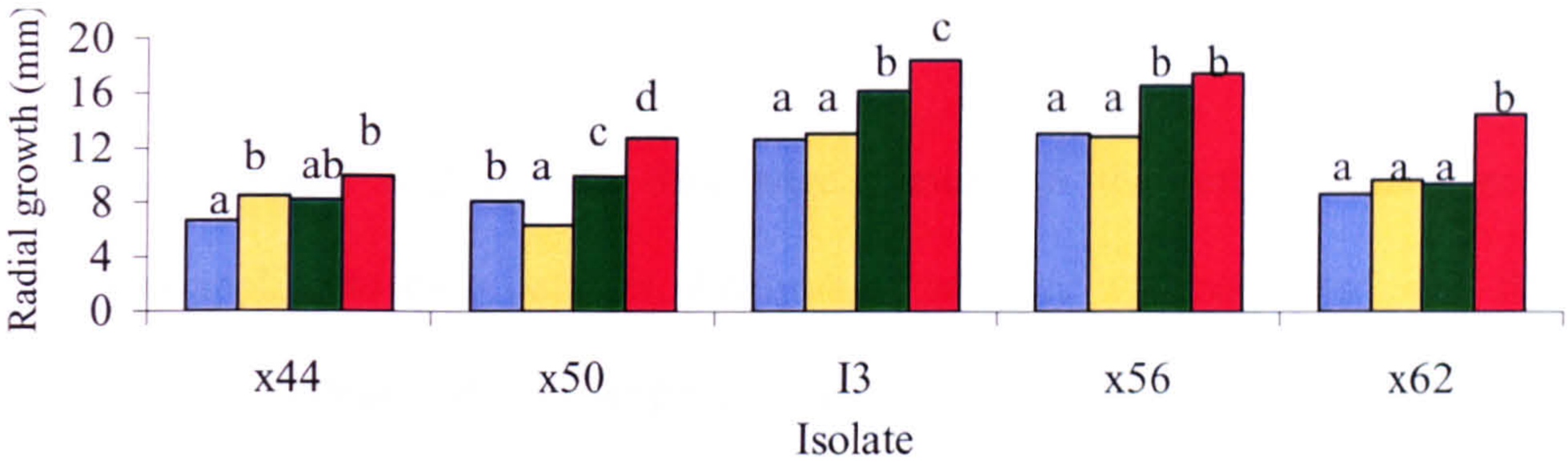
(c) 144h



d.f. = 171 S.E.M. = 0.63 L.S.D. (P=0.001) = 2.695 %cv = 13.7

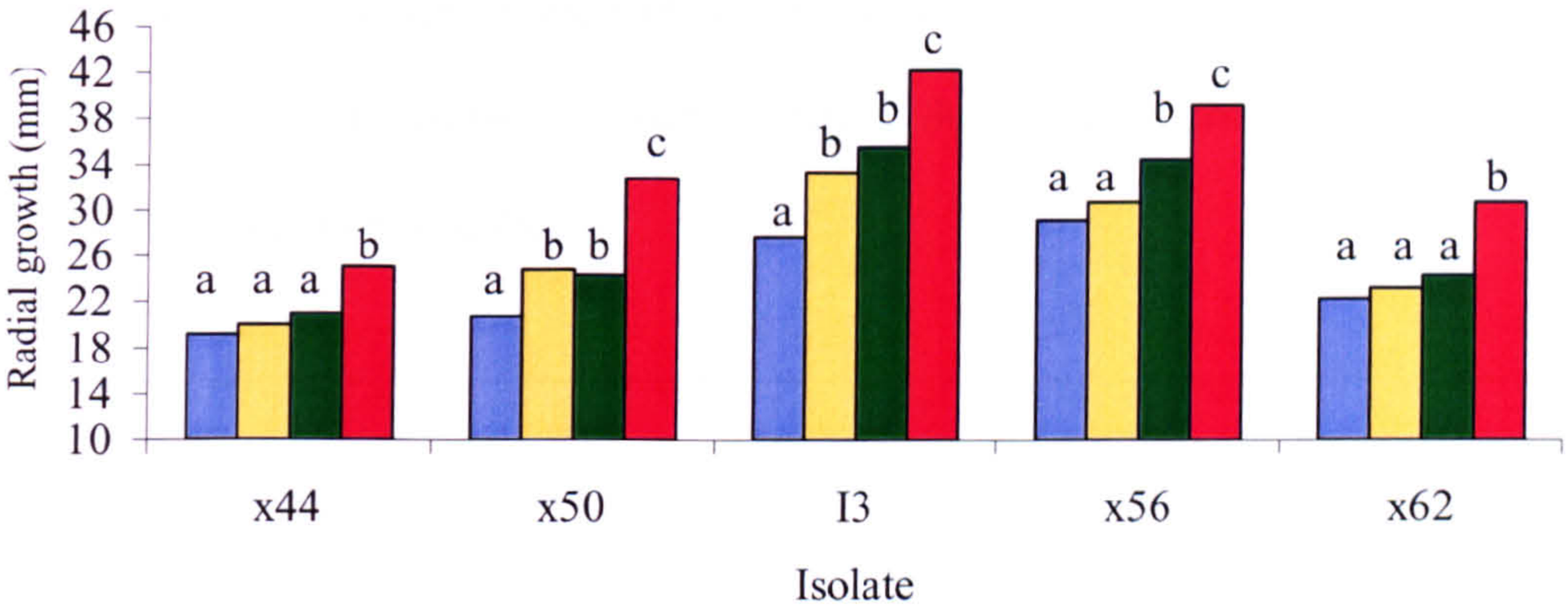
Figure 5.8 The radial growth of *R. solani* isolates grown on unamended water agar (■), water agar amended with leachate from pots containing silver sand only (■), water agar amended with leachates from potato plants (cv. Désirée) uninfested (■) or infested with *G. rostochiensis* (■) at (a) 48, (b) 96 and (c) 144 days after inoculation with *R. solani* isolates. Leachates were collected 8 days after *G. rostochiensis* juveniles were introduced. Bars with the same letter are not significantly different according to L.S.D. statistics.

(a) 48h



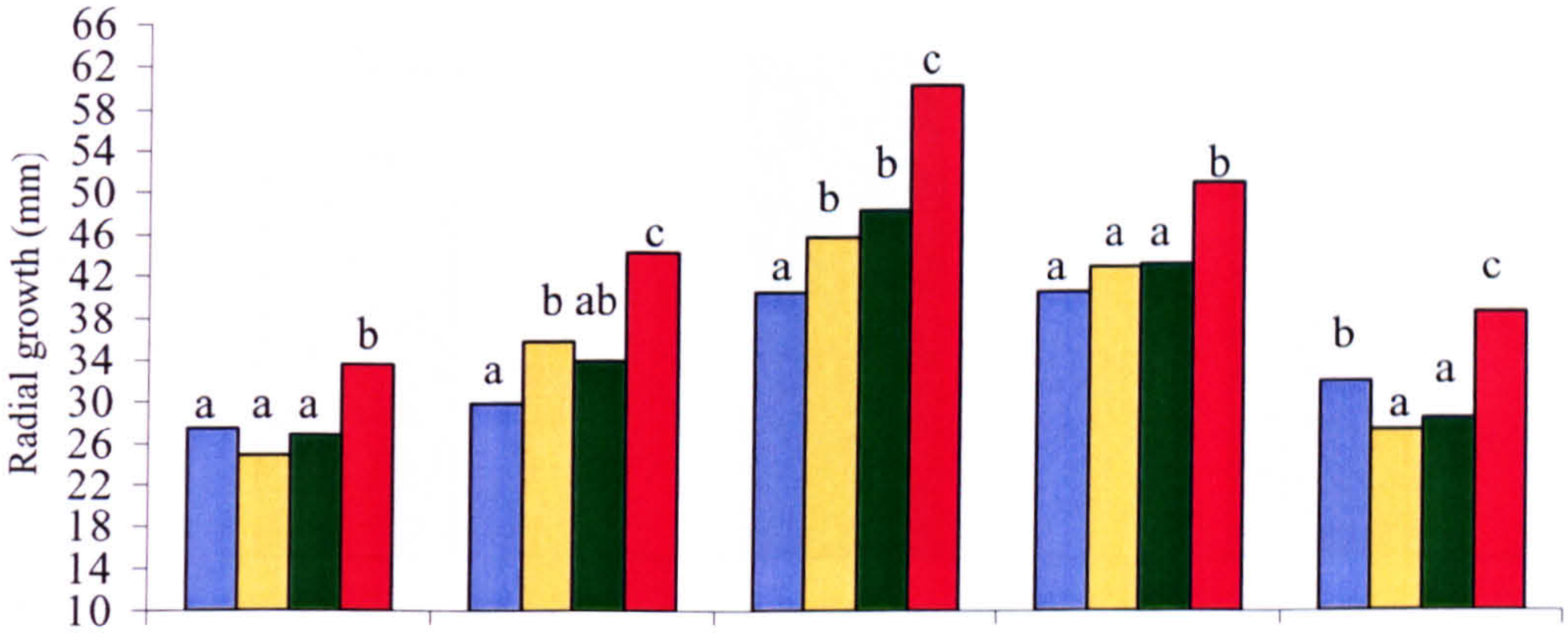
d.f. = 171 S.E.M. = 0.29 L.S.D. (P=0.001) = 1.729 %cv = 16.8

(b) 96h



d.f. = 171 S.E.M. = 0.49 L.S.D. (P=0.001) = 2.218 %cv = 9.0

(c) 144h

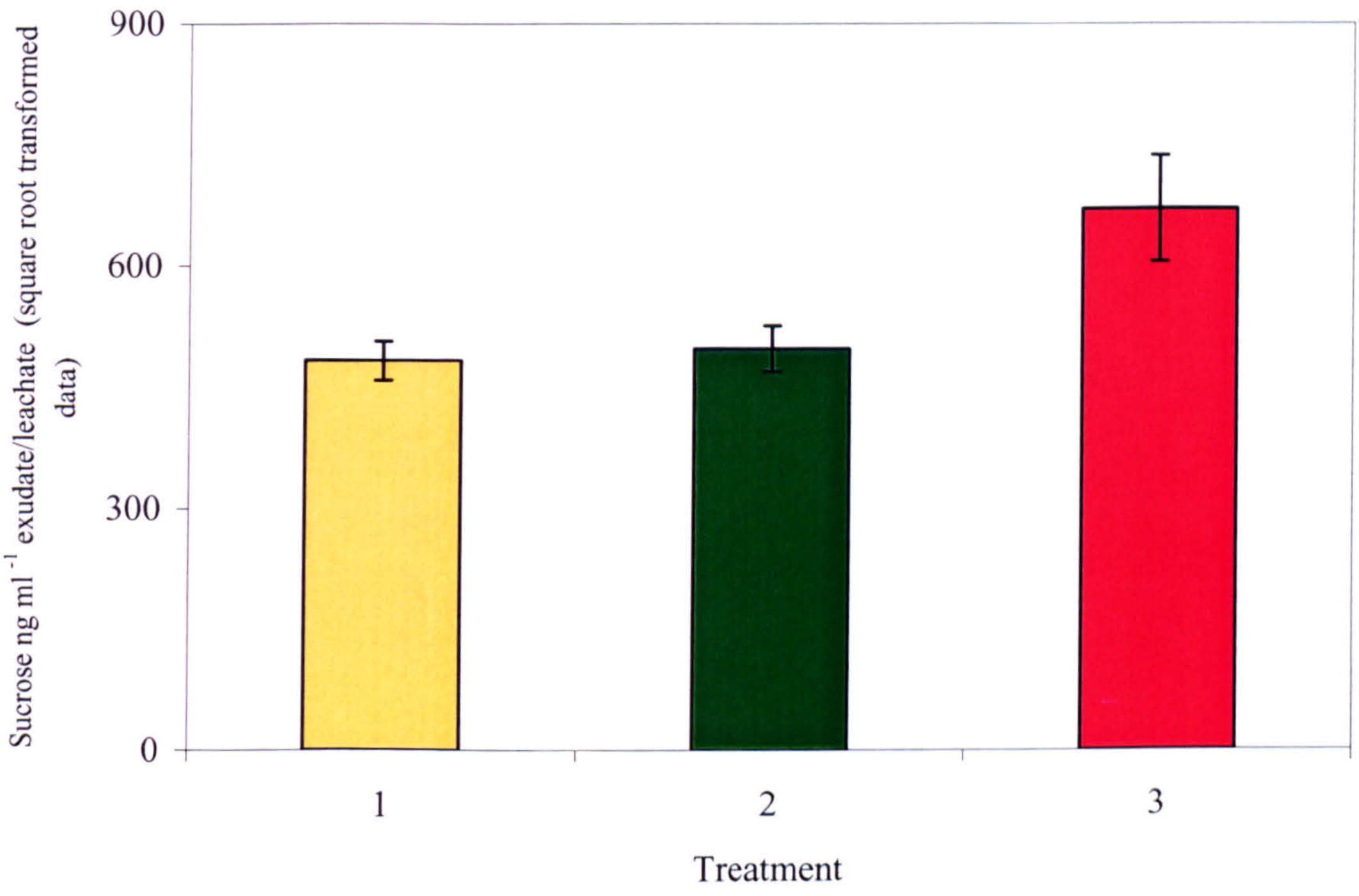


d.f. = 171 S.E.M. = 0.69 L.S.D. (P=0.001) = 2.770 %cv = 8.3

Figure 5.9 The radial growth of *R. solani* isolates grown on unamended water agar (■), water agar amended with leachate from pots containing silver sand only (■), water agar amended with leachates from potato plants (cv. Désirée) uninfested (■) or infested with *G. rostochiensis* (■) at 48 (a), 96 (b) and 144 (c) hours after inoculation with *R. solani* isolates. Leachates were collected 12 days after *G. rostochiensis* juveniles were introduced. Bars with the same letter are not significantly different according to L.S.D. statistics.

5.3.2.2 Analysis of sugar content in leachates

Since the glucose, fructose and sucrose data were skewed to the left, a square root transformation was applied to each data set. Analysis of variance was conducted with the explanatory variates, collection date and experimental treatment. Sucrose was found to be significantly higher in leachates from potato roots infested with *G. rostochiensis* than in leachates from potato plants uninfested with *G. rostochiensis* ($P<0.01$) (Figure 5.10). No significant differences were found between harvest dates. Glucose and sucrose content did not differ significantly between treatments.



d.f. = 26 S.E.M. = 0.025 %cv = 63.6

Figure 5.10 Mean sucrose content of 1) leachates from pots containing silver sand alone, 2) leachates from potato plants (cv. Désirée) uninfested with *G. rostochiensis* and 3) leachates collected from potato plants (cv. Désirée) uninfested with *G. rostochiensis* collected 4, 8 and 12 days after *G. rostochiensis* treatments were administered. Error bars denote the standard error of the mean

5.3.2.3 Analysis of nitrogen content in leachates

There were no significant differences between the total nitrogen content of leachates from *G. rostochiensis* infested or uninfested plants or in the leachates collected from pots containing sand alone.

5.3.2.4 Invasion of potato roots by *G. rostochiensis* juveniles

The invasion of potato roots by *G. rostochiensis* juveniles in experiment 2 is shown in Table 5.8. No juveniles were found in plants uninfested with *G. rostochiensis*. Figure 5.11 shows the distribution of juvenile stages recovered.

Table 5.8 The invasion of potato roots (cv. Désirée) by *G. rostochiensis* juveniles in controlled environment experiment 2

Treatment	Mean *G. rostochiensis* juveniles g^{-1} root	S.E.	%cv
Uninfested potato plants	0	0	-
Potato plants infested with *G. rostochiensis* juveniles	879.8	91.4	38.9

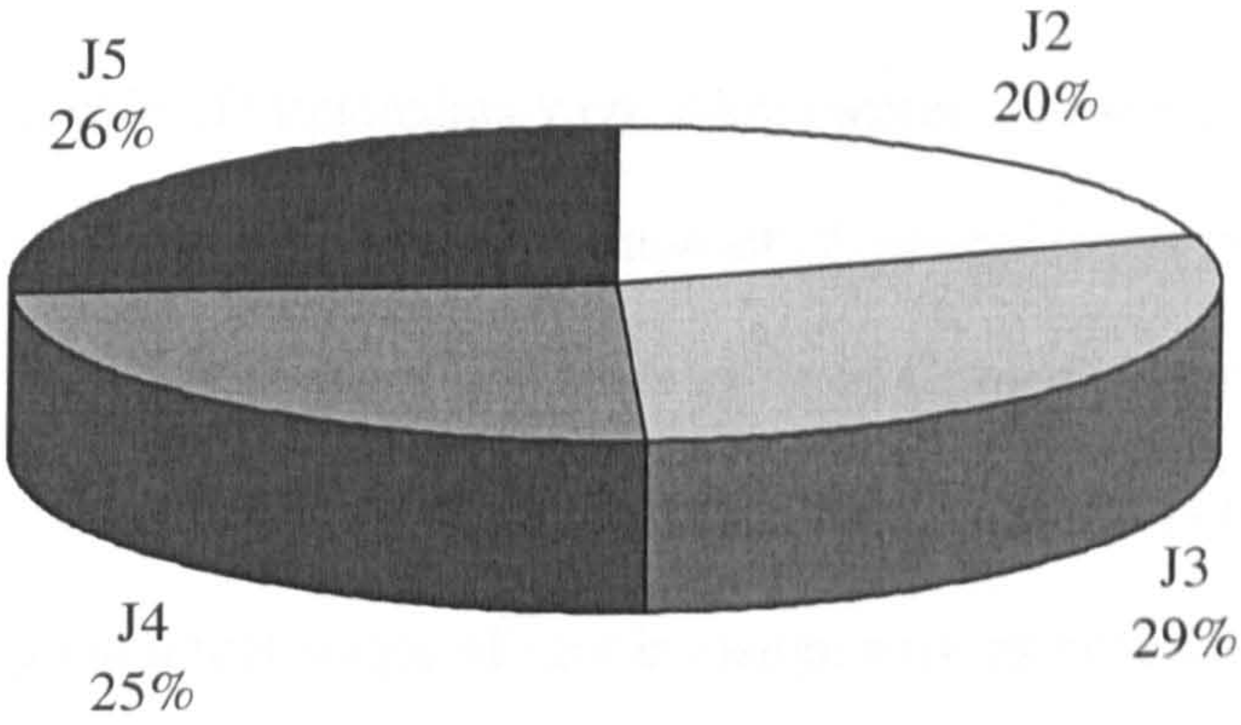


Figure 5.11 The mean distribution of *G. rostochiensis* juvenile stages (J2-J5) recovered from potato (cv. Désirée) roots, 12 days after their introduction to 2-week-old plants in experiment 2

5.4 Discussion

Plant root leachates are fundamental in stimulating the growth of microorganisms due to the release of organic compounds (Rovira, 1965; Bolton *et al.*, 1993; Grayston & Jones, 1996; Griffith *et al.*, 1999). Furthermore, the rate at which root exudates are released can be further increased by root injury (Bolton *et al.*, 1993). On this basis, several workers have hypothesised that root damage, caused by plant parasitic nematodes, may increase the release of root exudates and ultimately increase the attraction of soil-borne pathogens (Bergeson *et al.*, 1970; Van Gundy *et al.*, 1977).

The current work has clearly demonstrated an increase in the radial growth of the soil-borne fungus *R. solani* on media amended with root leachates from *G. rostochiensis* infested potato plants compared to media amended with root leachates from *G. rostochiensis* uninfested plants over two consecutive experiments. In the first experiment, the radial growth of *R. solani* was greater on media amended with potato root leachates from *G. rostochiensis* infested plants than on media amended with leachates from uninfested plants collected 4, 6, 8 and 12 days after the *G. rostochiensis* treatments were administered. However, no significant differences were observed 18 days after the introduction of *G. rostochiensis* juveniles. This may suggest the importance of different stages in the life cycle of *G. rostochiensis* in relation to the release of potato root leachates. These results indicate that *G. rostochiensis* juveniles modify root exudation during the initial stages of root invasion, such as penetration, migration through the root tissue and initialisation of feeding sites (see Chapter 1). Since very few J5 females were recorded in the juvenile analysis of harvested potato roots, the effects of female emergence on root exudation were not accounted for in this experiment.

In all of the *R. solani* radial growth assays conducted during experiment 1, the growth of *R. solani* was greatest on media amended with leachates collected from pots containing silver sand alone (potting medium control). This finding may relate to the weekly dose of fertiliser applied to all of the pots to sustain the potato plants during the experiment. Where potato plants were present, the fertiliser is likely to have been utilised by the plant whereas pots without plants may have retained higher quantities of fertiliser. Since positive interactions have been demonstrated between nitrogen sources and the growth of fungal pathogens (Solomon *et al.*, 2003), it could be hypothesised that the increased growth of *R. solani* observed in the current experiment was due to residual nitrogen. As a result of these findings fertiliser treatments were not administered in the second experiment.

The second experiment investigating the radial growth of *R. solani* on media amended with root leachates from *G. rostochiensis* infested or uninfested potato plants provided a much clearer picture. Each *R. solani* isolate grew more on media amended with root leachates from *G. rostochiensis* infested plants than on media amended with root leachates from uninfested plants. This trend was particularly pronounced when media was amended with root leachates collected 12 days after *G. rostochiensis* juveniles were introduced to potato plants. At 12 days after the introduction of juveniles there was a relatively equal split of the juvenile moults J2, J3, J4 and J5 recovered from potato roots. The relatively high proportions of J2 (20%) and J3 (29%) found at this time help support the earlier suggestion that *G. rosttochiensis* juveniles modify root exudates during the early satges of invasion. It should also be noted that although J5 juveniles were recovered at this date only 8% were female. This would, therefore, negate the potential effects of female emergence on root exudation.

In view of the modifications used in the second experiment, it could be reasonably expected that the *R. solani* radial growth experiments results might have greater definition than those recorded in the first experiment. This could be due to a number of reasons such as the higher number of *G. rostochiensis* juveniles used to infest potato plants, the higher volumes of leachate used to amend the agar or the reduction in filtering stringency.

Carbohydrate analysis of leachates collected during the second experiment indicated significantly higher concentrations of sucrose in leachates from potato plants infested with *G. rostochiensis* than leachates from uninfested plants. Glucose and fructose concentrations were also higher in leachates from plants infested with *G. rostochiensis* but were not significantly different from the leachates of uninfested plants. Since the growth and activity of microorganisms is limited by the availability of nutrients in agricultural soils (Nelson, 1990), the presence of higher sucrose concentrations coupled with the observations of increased *R. solani* radial growth may be critical in explaining the disease complex found between *G. rostochiensis* and *R. solani* in the field experiments of this project. The growth of *R. solani* in response to the nutrients within leachates has been previously observed (Reddy, 1980; Ritz, 1995; Ritz *et al.*, 1996). Reddy (1980) found a correlation between the decline of compounds such as sugars (sucrose and glucose) and amino acids within exudate from the hypocotyls of groundnut seedlings of different ages and the *in-vitro* growth of *R. solani*. In addition, Ritz (1995) illustrated that spatial densities of *R. solani* mycelium would correspond to spatial patterns of nutrient distribution. In the present study, there are indications that the relationship between *G. rostochiensis* invasion of potato roots and the infection of potato stolons by *R. solani* may be the consequence of modified root exudation caused by elevated root damage during the invasion of *G. rostochiensis* juveniles.

No significant differences were found between the total nitrogen content of root leachates from potato plants infested or uninfested with *G. rostochiensis*. In addition, the nitrogen content of potato root leachates did not differ significantly from leachates collected from pots containing sand alone. This result is surprising considering the number of workers who have found nitrogenous molecules in root leachates (Reddy, 1980; Kraffczyk *et al.*, 1984; Grayston *et al.*, 1996). However, it should be noted that the publications listed above relate to root leachates from plants other than potato. Although, considerable work has been undertaken on the constituents of root leachates in relation to the hatching process in potato cyst nematodes, the concentration of nitrogenous compounds in potato root leachates is seldom reported.

A possible explanation for the nitrogen results could be that the filtering process retained a proportion of nitrogenous macromolecules such as proteins. Further analyses using unfiltered root leachates from *G. rostochiensis* infested and uninfested potato plants would help clarify this hypothesis.

Van Gundy *et al.* (1977) investigated a similar hypothesis to that of the current investigation when examining interactions between *Meloidogyne incognita* and *R. solani* root rot disease on tomatoes. Root leachates collected from *M. incognita* infested tomatoes had higher total carbohydrate levels than uninfested tomatoes, 14 days after nematode introduction, which is in agreement with the present work. The authors suggest that this related to the time at which *R. solani* was attracted to *M. incognita* infested roots. At 14-21 days following nematode introduction, the carbohydrate content of root leachates from nematode infested tomato plants was lower and was associated with the development of sclerotia by *R. solani*. Increased nitrogenous compounds such as amino acids and proteins were found in leachates from nematode infested tomato plants 28 days after the introduction of *M. incognita*. This appeared

to coincide with the germination of *R. solani* sclerotia and the subsequent invasion of nematode galls. In the present work, no significant differences were found between the nitrogen content of root leachates from potato plants infested or uninfested with *G. rostochiensis* 4-12 days after nematodes were introduced. Later collections of root leachates from potato plants infested with *G. rostochiensis* may have shown a shift in nitrogenous compounds compared to root leachates from uninfested potato plants. However, there are distinct differences in the lifecycles of *G. rostochiensis* and *M. incognita*, which may have been reflected in the results of the current investigations and that of Van Gundy *et al.* (1977). Most significantly, *M. incognita* and other root-knot nematodes induce the formation of galls on the roots of their host plants, whereas root morphology is generally unaffected in plants infested by cyst nematodes such as *G. rostochiensis* (Endo, 1987).

In order to try and elucidate the effects of root exudates from nematode infested tomatoes on the development of *R. solani* root rot, Van Gundy *et al.* (1977) used a drip irrigation system to leach out the exudates from *M. incognita* infested tomato plants that were also co-inoculated with *R. solani*. With this treatment, symptoms of root rot did not develop. Moreover, if leachates collected from *M. incognita* infested tomato plants were applied to uninfested tomato plants inoculated with *R. solani*, root rot symptoms appeared. Tomato plants inoculated with *R. solani* alone did not develop symptoms of root rot. However, it is not clear from this work, whether a treatment was included to test the effect of root leachates collected from uninfested plants on tomato plants inoculated with *R. solani* alone. The inclusion of such a treatment would determine whether the root rot was incited by quantitative changes in the exudates (the volume of leachates applied) or qualitative changes in the leachates constituents caused by nematode invasion. It should also be noted that the drip irrigation system used to leach root exudates away might have produced unsuitable environmental conditions for *R.*

solani root rot to develop. For example, both Lootsma & Scholte (1997) and Kyritsis & Wale (2002) have shown that the development of *R. solani* stem canker diseases on potato is reduced in soils with increasing moisture contents.

Whilst these experiments have gone some way to explain the interaction between *G. rostochiensis* population densities and *R. solani* diseases on potatoes, further research is needed to support these findings. There is a requirement for radial growth experiments to be conducted with the present *R. solani* AG3 isolates on media amended with sucrose concentrations similar to those determined in the current experiments. Such experiments would identify whether the increased radial growth of *R. solani* isolates on media amended with leachates from *G. rostochiensis* infested potato plants was associated with the increased levels of sucrose identified. In addition, it would be useful to undertake more detailed studies of the leachate profile to determine whether other molecules such as amino acids, proteins, and organic acids differed during *G. rostochiensis* infestations. It would be interesting to know if any toxic compounds are found in root leachates such as the glycoalkoloids alpha solanine and alpha chaconine and if so, whether they differ in root leachates from *G. rostochiensis* infested and uninfested potato plants. Analytical apparatus such as high performance liquid chromatic analysis (HPLC), mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR) may prove useful in the future studies of root leachates as indicated by other researchers (Lugtenberg *et al.*, 1999; Fan *et al.*, 2001). If a relationship was determined between the profiles of root leachates from *G. rostochiensis* infested plants and the growth of *R. solani* isolates *in-vitro*, the subsequent investigative steps would be to examine the effects of such exogenous nutrients on the pathogenicity of the fungus *in-vivo*.

It should be noted that all of the *R. solani* radial growth studies were undertaken using dilutions of filtered root leachates. A possible alternative to the methods used would leave the root leachates unfiltered and undertake radial growth experiments on *R. solani* selective media (Ko & Hora, 1971; Castro *et al.*, 1988). This would reduce the potential loss of root leachate constituents during filtering, whilst keeping contamination to the minimum. Another strategy would be to inoculate known volumes of undiluted root leachates, amended with selected antibiotics and fungicides with *R. solani* and measure the mycelial dry weight after a known time period.

CHAPTER 6.0 – GENERAL DISCUSSION

CHAPTER 6.0 – GENERAL DISCUSSION

In 1931, Howard Fawcett presented a presidential paper at the 'Annual Meeting of the American Phytopathological Society' entitled 'The Importance of Investigations on the Effects of Known Mixtures of Microorganisms'. During his talk, Fawcett debated that plant pathologists were not getting the full pathological story by solely studying single organisms in isolation and stated that "Nature rarely works alone but most frequently with associations". Following this profound presentation, interest in disease complexes including those concerned with plant parasitic nematodes and fungi increased, especially during the 1960s and 1970s. Although a vast number of interactions between nematodes and fungi were identified, few were fully investigated. This is particularly true of the interaction between the potato cyst nematode *Globodera rostochiensis* and the pathogen *Rhizoctonia solani* on potatoes. Grainger & Clark (1963) and Mazurkiewicz-Zapalowicz & Waker-Wójciuc (1994) undertook preliminary glasshouse investigations looking at these organisms and suggested that a synergistic interaction occurred between the two organisms.

On the basis of existing literature, it was clear that a more comprehensive study was required to fully elucidate the interaction between *G. rostochiensis* and *R. solani* and their effects on the potato crop. In this project, both glasshouse and field experiments were undertaken to firstly establish whether an interaction exists between *G. rostochiensis* and *R. solani*. Subsequent investigations were undertaken to determine possible mechanisms behind the disease complex.

Two years of field experiments (Chapter 4) have revealed a number of relationships between *G. rostochiensis* infestation of potatoes and the development of disease symptoms by *R.*

solani. Most prominently, a positive linear relationship was consistently observed between the invasion of potato roots by *G. rostochiensis* and the infection of potato stolons (stolon canker) by *R. solani*. Results from 2000 suggested that the relationship between *G. rostochiensis* infestations and stolon canker symptoms was strongest at six weeks after planting potatoes. Similarly, a corresponding relationship was observed in 2001 at this time. The localised nature of the *R. solani* infections indicated that specific mechanisms were involved between the interacting organisms. In view of these observations, it was hypothesised that invading *G. rostochiensis* juveniles modified the rhizosphere of potato plants by causing an increased outflow of root constituents. Previous studies have suggested that plant root exudates play a role in attracting *R. solani* to its host plant (Kerr, 1956; Martinson, 1965). Consequently, it was thought that changes in root exudation occurring during the invasion of potato roots by *G. rostochiensis* might increase the severity of the *R. solani* infections.

Field studies in 2001 utilised a smaller and more compact plot design to explore the effects of interacting organisms on tuber yield and the development of black scurf on progeny tubers by *R. solani*. Black scurf was not found to relate to earlier stem canker symptoms or infestations of *G. rostochiensis*. Other workers (Hide *et al.*, 1989a; Simons & Gilligan, 1997a) investigating the epidemiology of *R. solani* have speculated on the importance of soil-borne inoculum in black scurf development. Furthermore, the experiments of Dijst (1990) suggested that black scurf was triggered by volatile exudates such as ethylene, released during crop senescence. Consequently, the relationship between earlier infections by *R. solani* or infestations by *G. rostochiensis* on potato plants and the later development of black scurf may be difficult to interpret due to the complexity of other epidemiological factors such as the presence of naturally occurring soil-borne inoculum of unknown inoculum potential and distribution.

A reduction of tuber yield in relation to the interaction between *G. rostochiensis* infestation of potato roots and the development of stolon infections was observed in one of the two years of field study. Yield reductions in response to the combined presence of *G. rostochiensis* and *R. solani* have also been reported by Grainger & Clark (1963).

In contrast to the field studies, the glasshouse experiments of this project (Chapter 3) did not show a direct interaction between *G. rostochiensis* and *R. solani* diseases. This disparity may relate to the lower densities of *G. rostochiensis* juveniles found to invade the roots of potato under glasshouse conditions. Furthermore, field studies examined the relationship between *G. rostochiensis* and *R. solani* over a much wider range of population densities than those seen in the glasshouse work. Both glasshouse experiments were limited by the method used to infest potting medium with *G. rostochiensis*. To elaborate further, higher *G. rostochiensis* population densities were unattainable due to the impractical number of cysts that would require manual counting. The alternative method of using field soil containing natural populations of *G. rostochiensis* was avoided, in order to prevent contamination from *R. solani* soil-borne inoculum. Although further development of the glasshouse studies may have produced clearer results, the field experiments of this project have already provided detailed information on this interaction within the natural soil environment. The experiences of field and glasshouse work reported in this project may also be relevant to other researchers who intend to undertake studies on interactions between plant parasitic nematodes and soil-borne pathogens.

In Chapter 5, several controlled environment studies were conducted to explore the hypothesis that damage or colonisation of potato roots by *G. rostochiensis* might result in quantitative or qualitative changes in the release of root exudates to subsequently affect the growth of *R.*

solani in the potato rhizosphere. To address this hypothesis, the growth of 5 *R. solani* (AG 3) isolates were compared on media amended either with root leachates from *G. rostochiensis* infested or uninfested potato plants at different time intervals after the introduction of the pest. In addition, the concentrations of sugars and nitrogen were determined in each of the leachate samples collected. In the first experiment, the growth of *R. solani* was higher on media amended with potato root leachates from *G. rostochiensis* infested plants than on media amended with leachates from uninfested plants collected 4, 6, 8 and 12 days after the *G. rostochiensis* treatments were administered. However, no significant differences were observed 18 days after the introduction of *G. rostochiensis* juveniles. In order to explore these effects further a second experiment was performed with a number of modifications. In accordance with the first experiment, the *R. solani* isolates grew faster on media amended with root leachates from *G. rostochiensis* infested potato plants than on media amended with root leachates from uninfested plants. This growth trend was particularly pronounced when the medium was amended with root leachates collected 12 days after *G. rostochiensis* juveniles were introduced to potato plants. At this time, a moderate proportion of the *G. rostochiensis* juveniles present in the roots were found to belong to the juvenile moults J2 and J3, which indicates that root leachates were modified during the earlier stages of juvenile invasion.

Carbohydrate analysis of leachates collected during the second experiment indicated significantly higher levels of sucrose in leachates from potato plants infested with *G. rostochiensis* than leachates from uninfested plants, whereas no significant differences were found in the total nitrogen content. The presence of higher sucrose levels coupled with the observations of increased *R. solani* growth may be critical in explaining the disease complex found between *G. rostochiensis* and *R. solani* in the field experiments of this project. Whilst further work would be required to fully determine the significance of increased sucrose levels

in root leachates from *G. rostochiensis* plants on infection by *R. solani*, many other studies have demonstrated the importance of exogenous carbohydrates in the attraction and growth of fungi such as *R. solani* (Reddy, 1980; Ritz, 1995; Ritz *et al.*, 1996). In addition, these results are also supported by the findings of Van Gundy *et al.* (1977) who observed that tomato root leachates collected from *M. incognita* infested tomatoes had higher total carbohydrate levels than uninfested tomatoes, 14 days after nematode introduction.

The majority of work in this project has concentrated on investigating synergistic interactions between *G. rostochiensis* and *R. solani*. However, findings from the field experiments (Chapter 4) have also indicated antagonistic effects of *R. solani* on *G. rostochiensis*. For example, negative linear relationships were found between mean stolon infections caused by *R. solani* and the multiplication rate of *G. rostochiensis*, suggesting that potato plants heavily infected with *R. solani* will have a lower number of female nematodes maturing into cysts. *Rhizoctonia solani* could be affecting *G. rostochiensis* multiplication by competing for nutrients and/or by causing disruption to the nematodes' feeding site. These results emphasise the complexity of association between nematodes and fungi on a mutual host and the potential for multiple outcomes between the interacting organisms.

This project has investigated an interesting component of ecology occurring between two organisms of economic significance in the potato crop. It has identified a damaging disease complex occurring between the potato cyst nematode *Globodera rostochiensis* and the fungus *Rhizoctonia solani* under natural field conditions. Moreover, it has gone some way to explain why the interaction might occur. With this in mind, it is anticipated that these findings will add to the pool of existing knowledge, assist in the framework of future management strategies and be used to advise those involved in the production of potatoes.

6.1 Proposed future research

Following the observations made during this project, the following areas for future work in the study of 'The *R. solani-G. rostochiensis* disease complex of potato' are recommended.

- **Studies investigating the interaction between *G. pallida* and *R. solani*:** The current project has focussed upon interactions between *G. rostochiensis* and *R. solani*. However, it would be useful to conduct comparative studies with *G. pallida*. In a recent survey of potato cyst nematodes in England and Wales (Minnis *et al.*, 2000) 67 % of soil samples containing potato cyst nematode were comprised of pure *G. pallida* populations. Since *G. pallida* has a distinctly longer period of hatch than *G. rostochiensis* (Haydock & Evans, 1998), there is a requirement to determine the significance of the prolonged period of juvenile invasion on the development of *R. solani* diseases.

- **The significance of *R. solani* anastomosis groups:** As outlined in Chapter 1, *R. solani* is comprised of a number of strains known as anastomosis groups (AG's). Although AG 3 is the most commonly occurring AG on potatoes (Anderson, 1982; Balali *et al.*, 1995; Virgen-Calleros *et al.*, 2000) a number of other groups are associated with potato diseases (Chapter 1). AG 8 is particularly interesting because it solely attacks plant roots causing stunted growth. Given that the current project has indicated the importance of rhizosphere modifications in the interaction between *G. rostochiensis* and *R. solani* AG 3, interactions between *G. rostochiensis* and *R. solani* AG 8 may produce an acute disease complex since both organisms share a closer ecological

niche. Further studies would compare the effects of combined inoculations of *G. rostochiensis*, *G. pallida* and different *R. solani* AG's on potato.

- **Further investigations on the mechanism behind the *G. rostochiensis*-*R. solani* disease complex of potato:** The present study has provided indications that leachates from *G. rostochiensis* infested plants increase the growth of *R. solani in-vitro*. In addition leachates from *G. rostochiensis* infested plants were found to contain higher concentrations of sucrose. However, there is a requirement for radial growth experiments to be conducted with the present *R. solani* AG3 isolates on media amended with sucrose concentrations similar to those determined in the current experiments. Such experiments would identify whether the increased radial growth of *R. solani* isolates on media amended with leachates from *G. rostochiensis* infested potato plants was associated with the increased levels of sucrose identified.

  In addition, it would be useful to undertake more detailed studies on the leachate profile to determine whether other molecules such as amino acids, proteins, and organic acids differed during *G. rostochiensis* infestations. It would be interesting to know if any toxic compounds are found in root leachates such as the glycoalkoloids alpha solanine and alpha chaconine and if so, whether they differ in root leachates from *G. rostochiensis* infested and uninfested potato plants.

- **Studies investigating potential management strategies for the *G. rostochiensis* and *R. solani* complex:** Investigations should be undertaken to determine the most suitable methods to implement management strategies for the dual control of *G. rostochiensis* and *R. solani*. In particular, preliminary work has indicated the potential for using

granular nematicides to disrupt the disease complex (Back *et al.*, 2002). However, where mycophagous organisms such as springtails and mites are present, the application of granular nematicides may have the reverse effect, as seen by Hofman *et al.* (1991).

- **Modelling the interaction between *G. rostochiensis* and *R. solani*:** Few studies on interactions between plant parasitic nematodes and soil-borne pathogens have adopted a mathematical modelling approach. By modelling the interaction, the effect of prevailing environmental conditions, agronomic practice and other organisms could be taken into account to further improve our knowledge of this ecological niche. In addition the model could be used to forecast disease, allowing management strategies to be planned accordingly.

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APPENDICES

Water agar (WA)

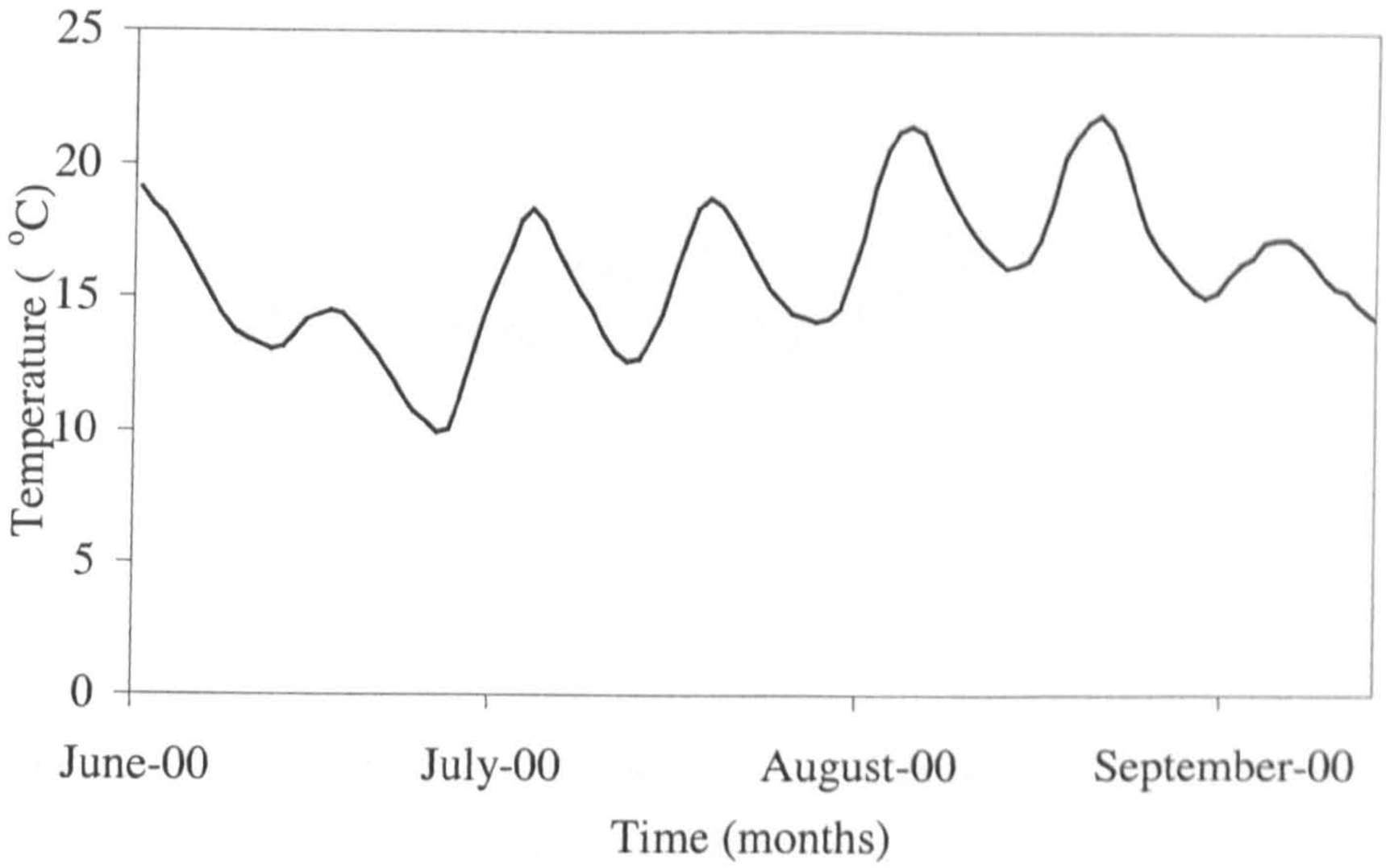
- 10g Agar No.2 (Lab M, Topley house, Wash Lane, Bury, England, BL9 6AU)
- 1l distilled water
- 2.5 ml of streptomycin sulphate solution (1g streptomycin sulphate in 20 ml sterile distilled water). Streptomycin was applied to the molten agar following cooling (45-55°C)

Potato dextrose agar (PDA)

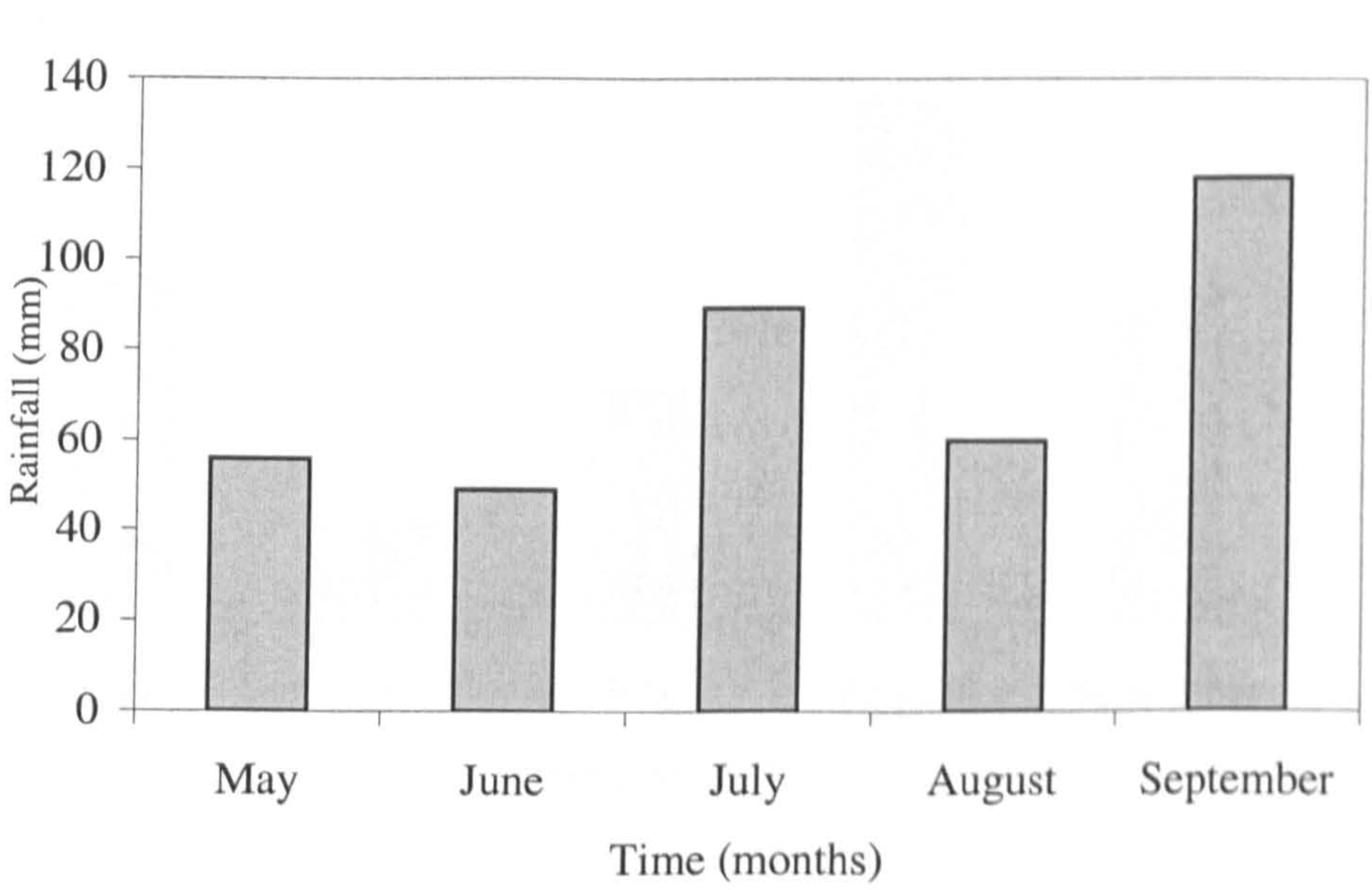
- 39g potato dextrose agar (Lab M, Topley house, Wash Lane, Bury, England, BL9 6AU)
  - 4g/l potato extract
  - 20g/l dextrose
  - 15g/l agar no. 1
- 1l distilled water
- 2.5 ml of streptomycin sulphate solution (1g streptomycin sulphate in 20 ml sterile distilled water). Streptomycin was applied to the molten agar following cooling (45-55°C)

Appendix 1 Details of agar based media used for culturing *Rhizoctonia solani*

a)

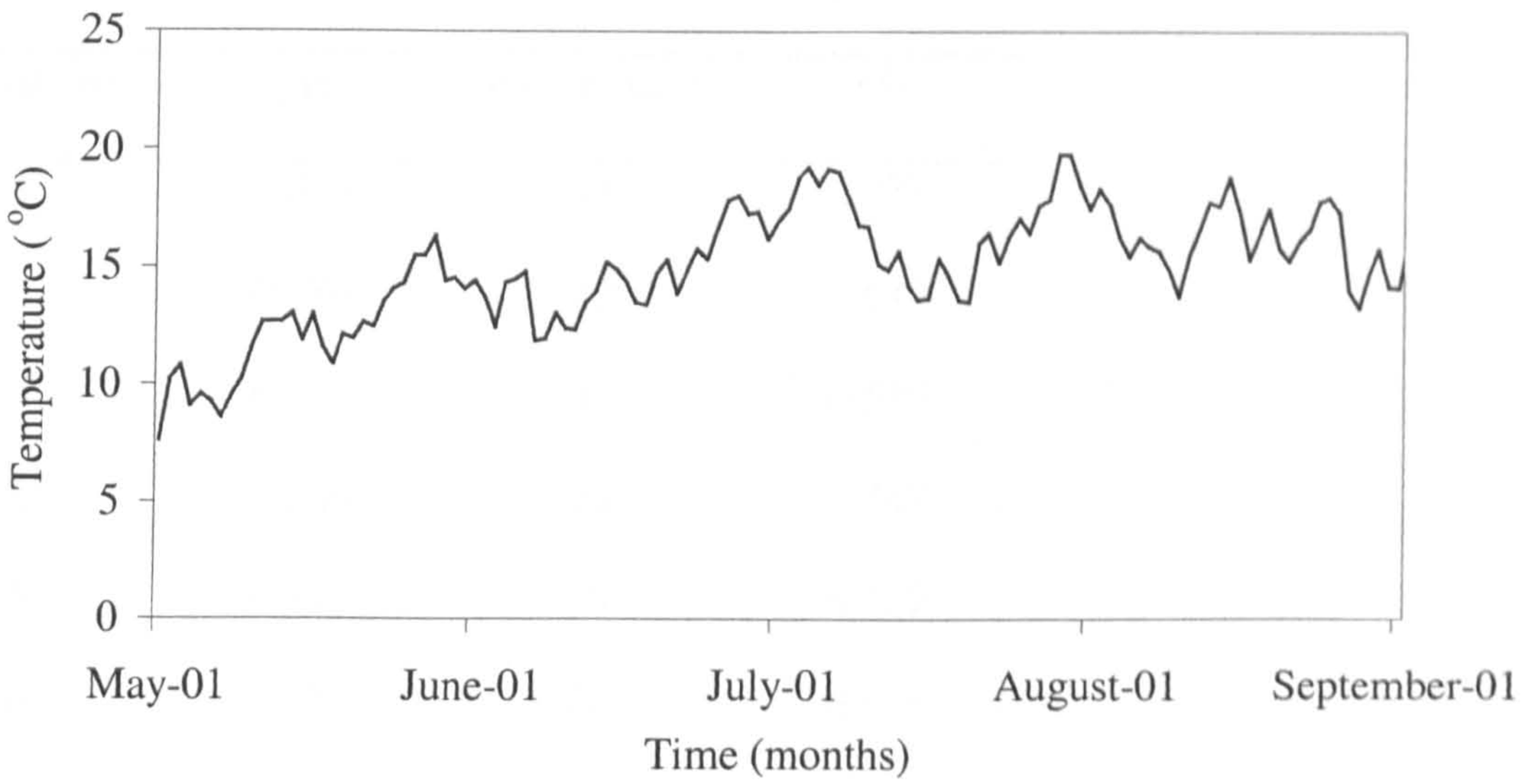


b)

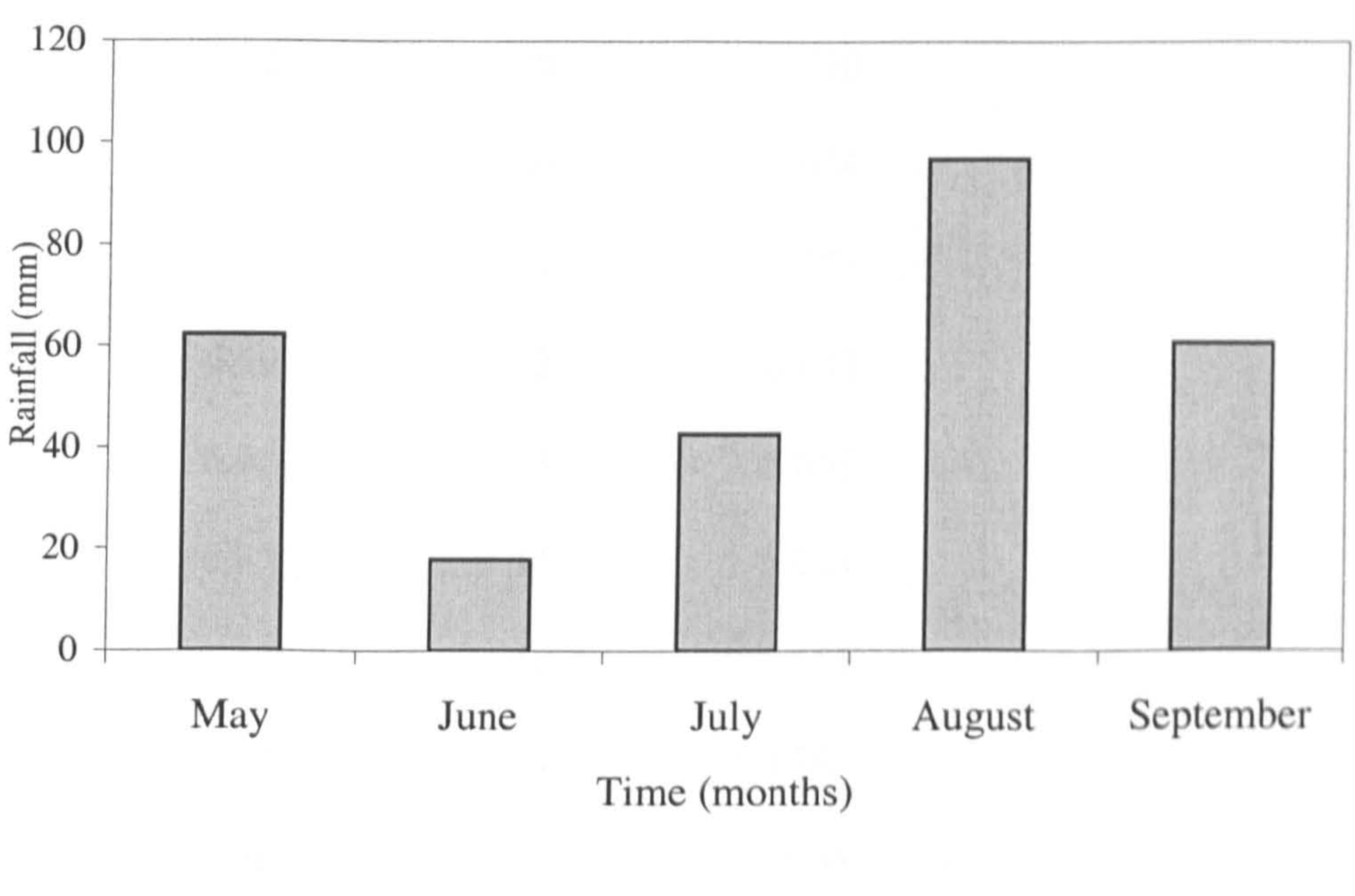


Appendix 2 Soil temperature (a) and total monthly rainfall (b) recorded during field experiment 2000

a)



b)



Appendix 3 Soil temperature (a) and total monthly rainfall (b) recorded during field experiment 2001

Appendix 4 Measurements of soil pH determined two weeks after planting field experiment 2000

Plot number	pH	Plot number	pH
1	6.346	**21**	6.737
2	6.486	**22**	6.619
3	6.485	**23**	6.559
4	6.586	**24**	6.747
5	6.417	**25**	6.719
6	6.439	**26**	6.658
7	6.555	**27**	6.633
8	6.640	**28**	6.789
9	6.328	**29**	6.720
10	6.444	**30**	6.614
11	6.540	**31**	6.756
12	6.699	**32**	6.847
13	6.481	**33**	6.857
14	6.375	**34**	6.744
15	6.425	**35**	6.781
16	6.548	**36**	6.786
17	6.085	**37**	6.908
18	6.035	**38**	6.886
19	6.338	**39**	6.774
20	6.363	**40**	6.784

Appendix 5 Measurements of soil pH determined two weeks after planting field experiment 2001

Plot number	pH	Plot Number	pH	Plot Number	pH
1	6.149	**25**	6.215	**41**	6.303
2	6.201	**26**	6.304	**42**	6.244
3	6.296	**27**	6.316	**43**	6.430
6	6.382	**28**	6.188	**44**	6.173
7	6.211	**29**	6.420	**45**	6.320
8	6.271	**30**	6.305	**46**	6.241
11	6.379	**31**	6.169	**47**	6.263
12	6.332	**32**	6.053	**48**	6.168
13	6.374	**33**	6.159	**49**	6.168
15	6.224	**34**	6.123	**50**	6.286
17	6.258	**35**	6.245	**51**	6.230
18	6.331	**36**	6.228	**52**	6.277
19	6.154	**37**	6.135	**53**	6.217
21	6.275	**38**	6.199	**54**	6.406
22	6.467	**39**	6.252	**55**	6.220
24	6.357	**40**	6.077	**56**	6.156